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CLINICAL AND EXPERIMENTAL

THE THYROID GLAND AND HEMATOPOIESIS*

II. THE EFFECT OF THYROID EXTRACT, LIVER EXTRACT, AND IRON ON THE ANEMIA OF MYXEDEMA

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THOUGH a moderate degree of anemia usually accompanies myxedema, there is considerable disagreement as to its type and to its proper treatment. Experimentally, we have found that the anemia is characterized by hyperchromia and macrocytosis.[1] Our purpose was to study the effect of the administration of thyroid extract, liver extract, and iron on this anemia of myxedema. In addition, it occurred to us that if this experimental macrocytic anemia of myxedema responded to liver extract, perhaps a method suitable for the standardization of liver extract could be developed.

EXPERIMENTAL METHODS

A total of 67 albino rabbits, approximately six months old was used. Because of the variability in normal blood counts, it was necessary to have a sufficiently large number of blood counts for statistical analysis. For the most part, the variations in normal counts were in close agreement with the findings of other investigators.[2-5] Of our 67 animals, 41 were subjected to total thyroidectomy, under local anesthesia, and the remainder used as normal controls. The operative procedure, care and feeding, weight records, and the hematologic technique has already been described.[1] Reticulocyte counts were made from cover slip smears stained with a 1 per cent alcoholic solution of

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brilliant cresyl blue. The percentage of reticulocytes was determined by counting 500 erythrocytes. After operation, three to five months were allowed to lapse before any experimental feedings were given. As in our preliminary observations, the first two months following thyroidectomy were characterized by a progressive decrease in the amount of hemoglobin and the number of erythrocytes, associated with a gradual increase in the mean corpuscular hemoglobin and the mean corpuscular volume of the erythrocytes (Figs. 1*A*, *B*). In addition, there was a gradual increase in weight and the development of signs of myxedema. From the third to fifth month, the amount of hemoglobin increased slightly from an average of 64 to 70 per cent. Following thyroidectomy in the rabbit, a moderate leucocytosis developed. We are not prepared at this time to consider the question of possible changes in the differential counts.

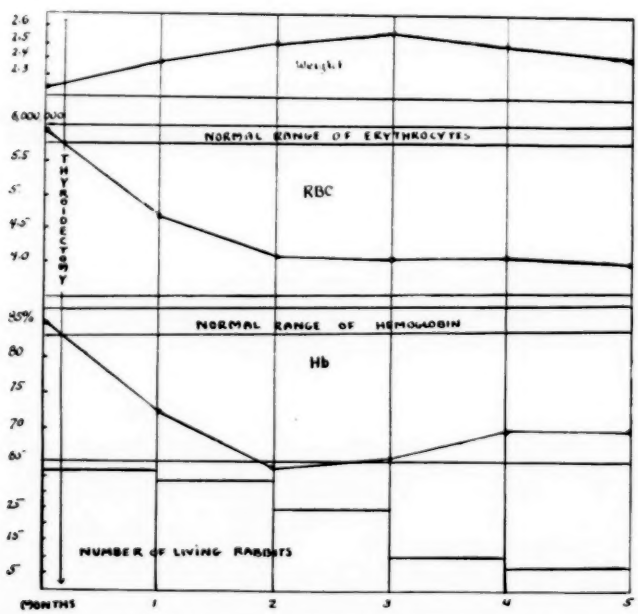

Fig. 1*A*.—The anemia of myxedema.

Each observation period lasted twenty-eight days. The first week served as a control period during which time hemoglobin determinations, erythrocyte, leucocyte, and differential counts were made on the first and sixth days. The weights and reticulocyte counts were determined daily. On the seventh day, the various preparations were first given and continued for three days in the group receiving liver, and for ten days in the groups receiving iron and thyroid extract. Liver extract* was given both intravenously and intramuscularly. Thyroid extract,* as an aqueous suspension, and iron and ammonium citrate, in a 50 per cent solution, were administered through a stomach tube during a fasting period. Daily weights and reticulocyte determinations were continued during the feeding period and differential blood counts were made

*The liver extract was supplied through the courtesy of Lederle and Co.; the thyroid extract by Armour and Co.

on the tenth and twenty-first day after the start of the test period. A number of the myxedematous animals and their normal controls were killed before, during, and after the experimental feedings, or injections, and complete autopsies with microscopic sections were made. The correlation of these bone marrow findings with the degree of response of the peripheral blood to the different feedings will serve for the basis of a later report. No residual thyroid tissue was found in any of the killed myxedematous animals.

EXPERIMENTAL OBSERVATIONS

The Response to Thyroid Extract.—Five normal and three thyroidectomized rabbits were given 2 gr. of thyroid extract daily for a ten-day period. In the normal group, there was a slight fall in the weights during the period of

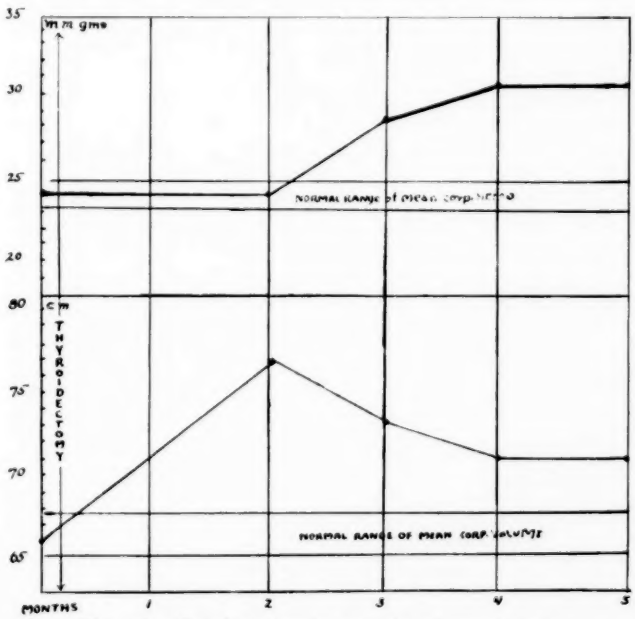

Fig. 1*B*.—The hematocrit, anemia of myxedema

thyroid ingestion. There was no significant change in either the reticulocytes or the hemoglobin and erythrocyte counts (Fig. 2*A*). In the thyroidectomized animals, thyroid extract caused the reticulocytes to rise from an initial 1.3 per cent to 12.0 per cent on the fifth day, followed by a sharp and sustained rise in both hemoglobin and erythrocyte levels. During thyroid feeding, the thyroidectomized animals showed a more striking and rapid weight loss than observed in the normal control (Fig. 2*B*).

In the second series of thyroid feedings, 6 normal and 6 thyroidectomized animals were given daily doses of 4 gr. of the extract. The normal group of this series was unsatisfactory as no one of the animals could be carried to the end of the observation period because of the appearance of marked muscular weakness, diarrhea, rapid weight loss, and death. Though the reticulocytes rose to 11.2 per cent on the eighth day, the later effect on the hemoglobin and

erythrocytes could not be determined (Fig. 3*A*). In the 6 thyroidectomized rabbits, the daily dose of 4 gr. of thyroid extract was followed by a slow, but

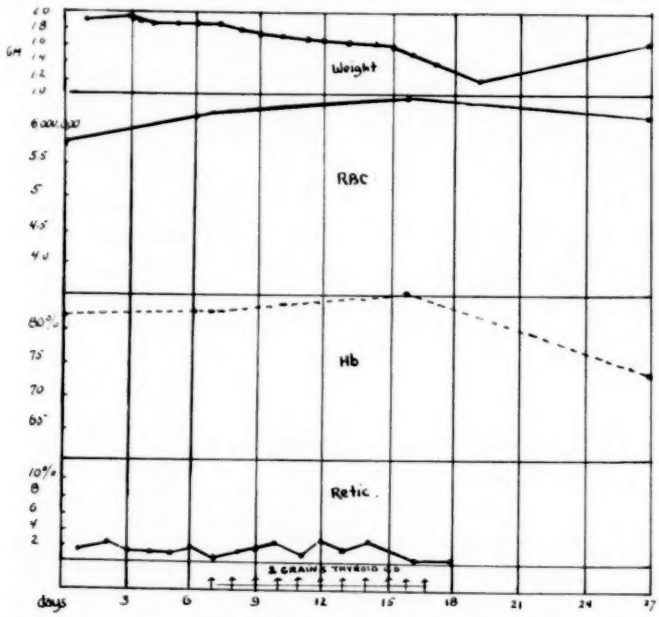

Fig. 2*A*.—Five normal rabbits. Response to 2 gr. of thyroid.

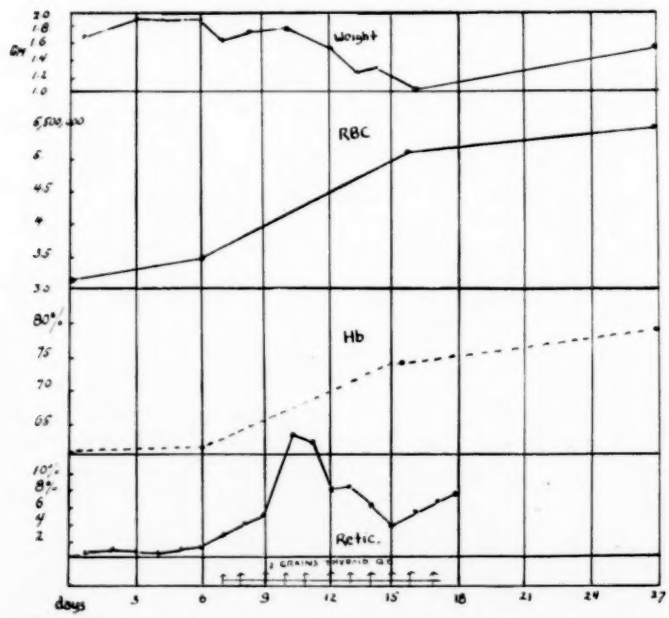

Fig. 2*B*.—Three thyroidectomized rabbits. Response to 2 gr. of thyroid.

sustained rise of the reticulocytes from an initial 2.1 per cent to 9.2 per cent that lasted through a twelve-day period. There was a sharp rise in hemoglobin and the number of erythrocytes to normal levels (Fig. 3*B*).

In the third series, 6 gr. of thyroid extract was given for a ten-day period. Of the 5 normal animals, only 2 survived the full observation period and in

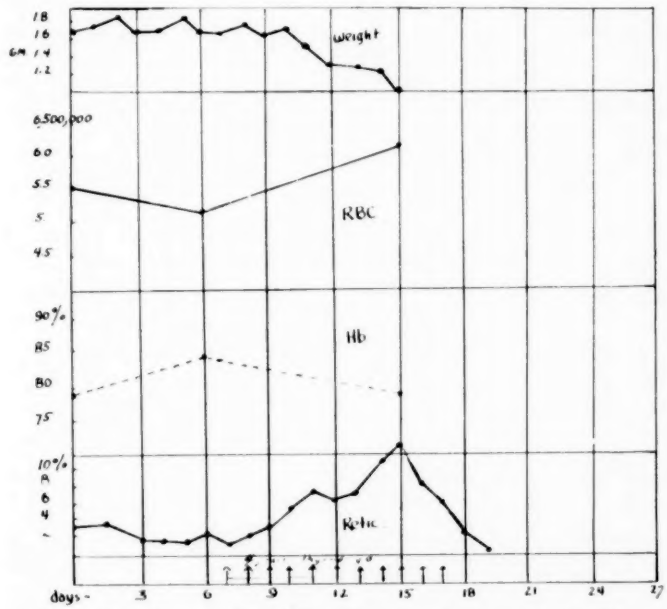

Fig. 3A.—Six normal rabbits. Response to 4 gr. of thyroid.

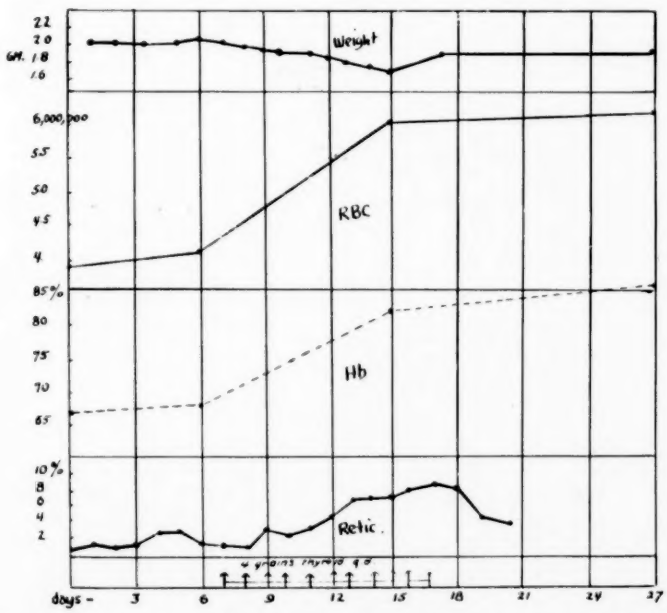

Fig. 3B.—Six thyroidectomized rabbits. Response to 4 gr. of thyroid.

neither was there any significant effect of the reticulocytes or the blood count (Fig. 4A). In the ten myxedematous animals, thyroid feeding resulted in a moderate reticulocyte response from the control 2.4 per cent to 7.5 per cent

on the seventh day, followed by a moderate increase in hemoglobin and erythrocyte counts (Fig. 4*B*). The 4 to 6 gr. daily dose of thyroid extract

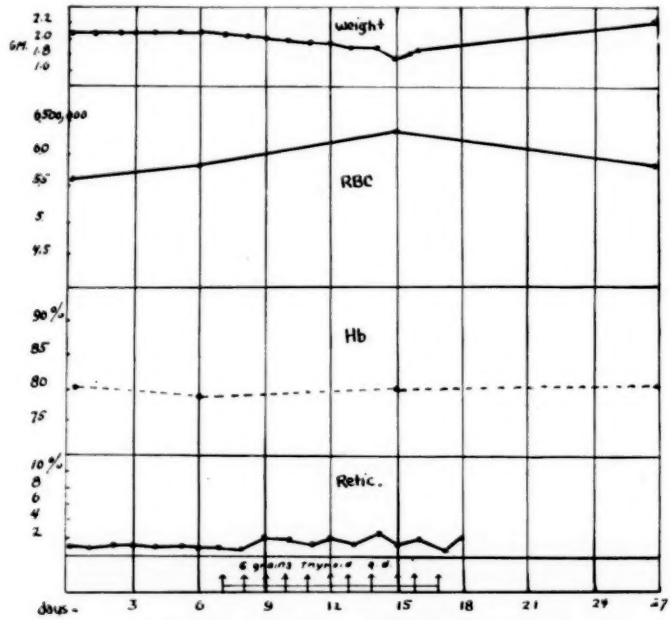

Fig. 4*A*.—Five normal rabbits. Response to 6 gr. of thyroid.

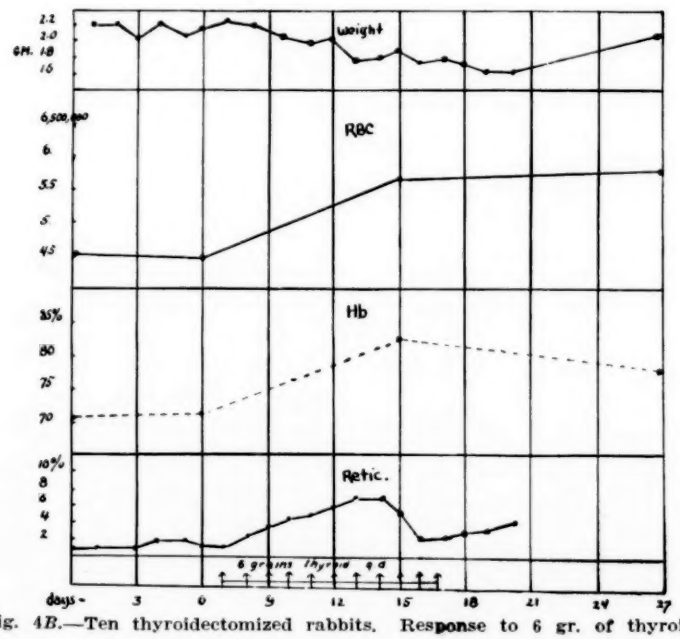

Fig. 4*B*.—Ten thyroidectomized rabbits. Response to 6 gr. of thyroid.

was not well tolerated in the normal control animals. Their anemic, myxedematous litter mates, however, showed no apparent ill effects.

The Response to Liver Extract.—In this experiment, intravenous and intramuscular liver extract was given to see what effect it might have on this

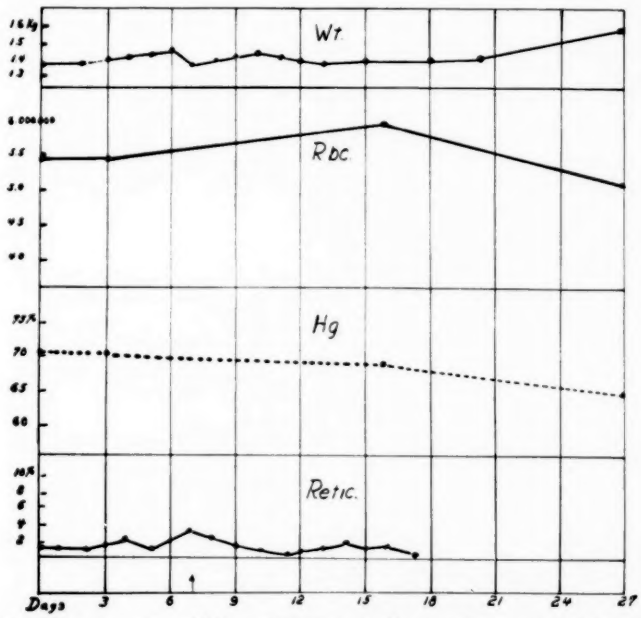

Fig. 5*A*.—Two normal rabbits. Response to liver extract given intravenously

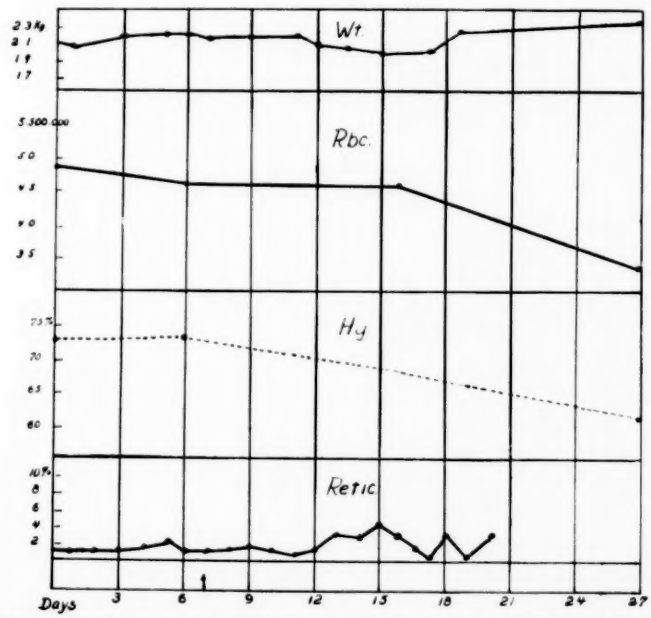

Fig. 5*B*.—Three thyroidectomized rabbits. Response to liver extract given intravenously.

macrocytic anemia of myxedema. In the intravenous group, 2 normals and 3 thyroidectomized rabbits were each given one dose of 0.5 c.c. of the extract. In neither the normal controls nor the myxedematous animals was there any

significant effect on either the reticulocytes or the blood counts. In fact, the anemia of the thyroidectomized animals continued its slowly progressive course in spite of the liver extract (Figs. 5*A*, *B*).

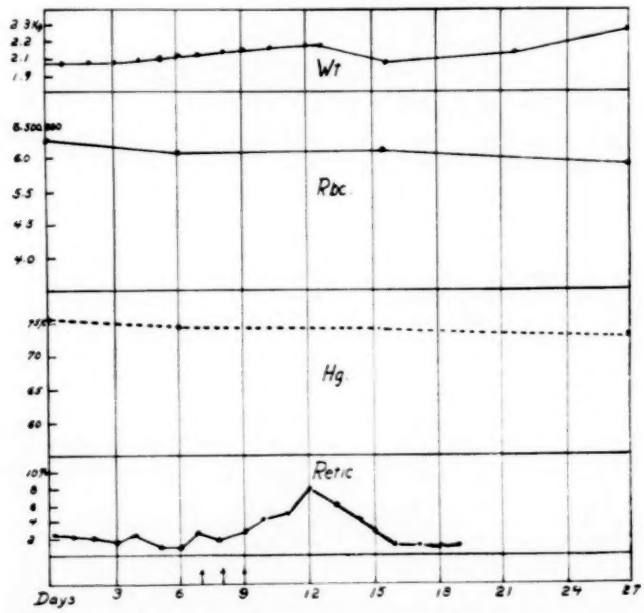

Fig. 6*A*.—Four normal rabbits. Response to liver extract given intramuscularly.

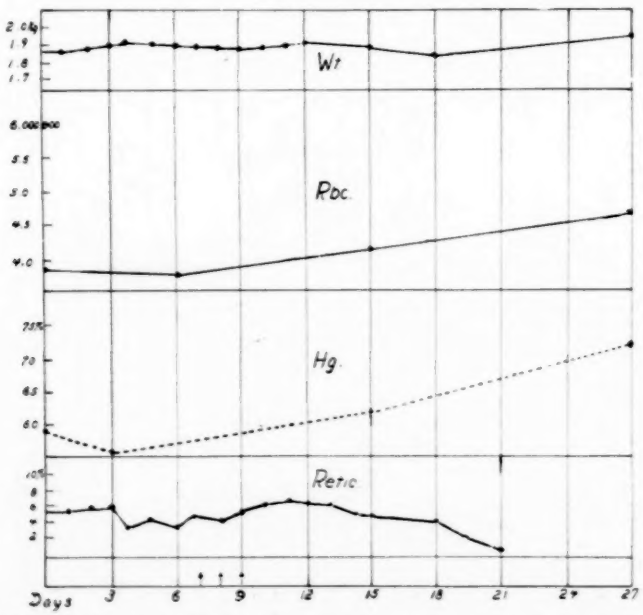

Fig. 6*B*.—Eight thyroidectomized rabbits. Response to liver extract given intramuscularly.

In the intramuscular group, 4 normal and 8 thyroidectomized animals were given 1.0 c.c. of liver extract on three successive days. The normal ani-

mals showed an increase of reticulocytes from 2.0 per cent to 8.4 per cent on the sixth day, but there was no later increase in hemoglobin or erythrocyte

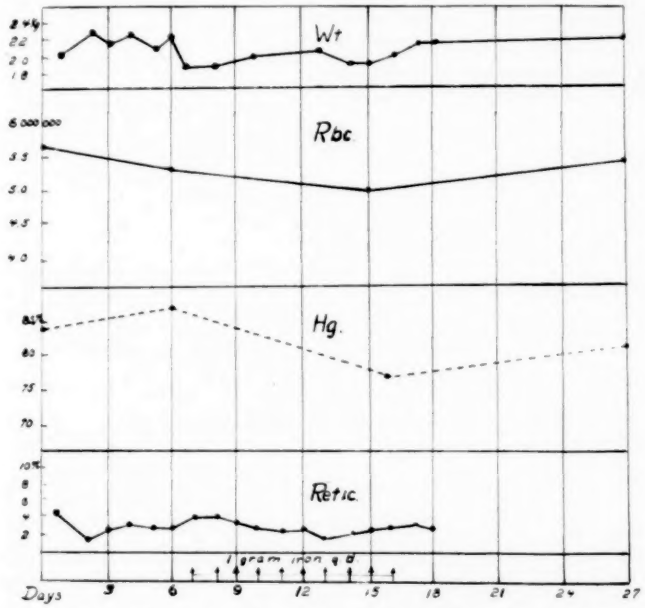

Fig. 7A.—Three normal rabbits. Response to 1 gm. of iron.

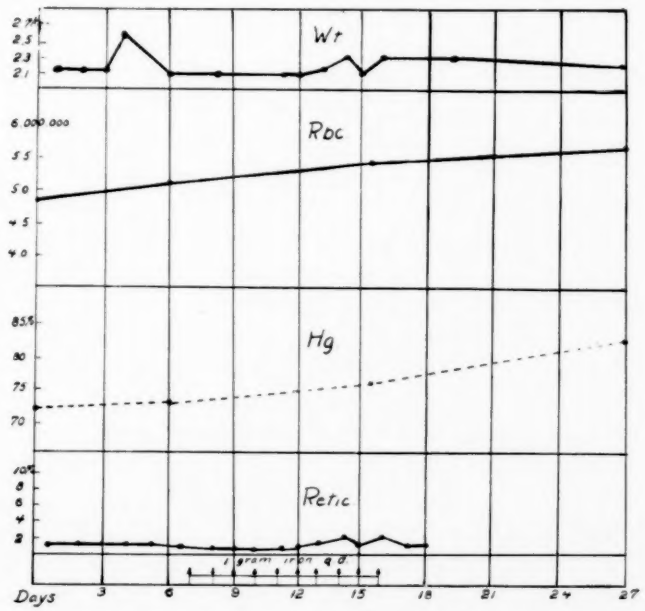

Fig. 7B.—Seven thyroidectomized rabbits. Response to 1 gm. of iron.

values (Fig. 6A). The control reticulocytes of the thyroidectomized animals were slightly higher than the usual level (4.3 per cent). Following the intra-

muscular liver extract, the reticulocytes increased to 6.5 per cent on the fifth day, followed by a slight, but definite, increase in both hemoglobin and red counts (Fig. 6*B*).

The Response to Iron.—A daily dose of 15 gr. of iron and ammonium citrate was given to 3 normal and 7 thyroidectomized rabbits for a period of ten days. In the normal animals, there was no increase in the reticulocytes or significant alteration in the red blood cell count (Fig. 7*A*). In the anemic, myxedematous animals, the iron produced no effect on the reticulocytes, although the erythrocytes and hemoglobin showed a very slight rise (Fig. 7*B*).

DISCUSSION

Esser[6] was the first to report an anemia by total thyroidectomy in rabbits. Orr[7] also found an anemia developed in the adult thyroidectomized rabbit, but concluded there was no significant change in the resultant color index. On the other hand, Kunde and others[8] working with cretin rabbits, found a progressive anemia characterized by a greater than normal diameter of the erythrocytes and a color index of greater than one. In our preliminary report,[1] and in the present study, these latter findings were corroborated.

The mechanism in the production of the anemia of myxedema remains unknown. The question naturally arises as to whether a plasma volume shift with resulting hydremia could possibly account for the blood changes in myxedema. The belief that the anemia in myxedema is real and not apparent is based on the work of Friedlander and others,[9] who found a sharp decrease in blood volume following thyroidectomy in male cats. Moreover, Thompson[10] concluded from his clinical observations that (1) in myxedema, as the weight increased, the total plasma volume decreased, and (2) in myxedema, there was frequently a decrease of the total blood volume by 25 per cent. This total plasma decrease in myxedema should tend to cause concentration of the cellular content of the blood, but instead, an anemia develops. In order to corroborate these findings, we attempted blood and plasma volume determinations in our series of operated and control rabbits, but the well-known experimental errors of these procedures render the results somewhat inconclusive.

Most authors feel that the anemia of myxedema is the result of the effect on the bone marrow of the sluggish oxidation that presumably occurs in all tissues.[11-13] That the thyroid gland has a direct influence on hematopoiesis is further evidenced by the consistent response of the anemia of myxedema to thyroid extract. Kunde and others[8] found histologic evidence of bone marrow stimulation in her cretin rabbits, and suggested that "the stimulus to metabolism initiated by large amounts of thyroid causes an acceleration of growth in the cells of the bone marrow, beyond the ability of the anemic animal to supply proper amounts of oxygen. This would result in a temporary oxygen want comparable to that which occurs upon ascending to high altitudes." We are not yet prepared to make any conclusions concerning the bone marrow findings of our animals, but such a study should prove of interest.

Though we found no significant effect from thyroid extract on the blood of our normal animals, Lim and others[14] found evidence of a stimulating

effect in the bone marrow. Kunde and others[8] noted little or no reticulocyte response in her normal animals, and only a slight temporary increase in the erythrocytes and hemoglobin.

Though liver extract has been tried unsuccessfully in the clinical treatment of the anemia of myxedema,[15] we find in rabbits evidence of moderate reticulocyte activity following its intramuscular use. However, since the increased reticulocytes also occurred in the normal control rabbits, it seemed to us that there was nothing specific in the action of liver extract in the anemia of myxedema. Moreover, the use of the anemic, myxedematous rabbit as a test animal in attempting to determine the degree of liver potency does not give uniform results.

Iron, in combination with thyroid extract, has been used clinically in the treatment of the anemia of myxedema to accelerate the regeneration of the blood cells. Experimentally, however, iron had no appreciable effect on the blood of the myxedematous animals. The discrepancy between these clinical and experimental results following the use of iron, undoubtedly is due to the differences in the type of the anemia that is found. In the totally thyroidectomized rabbit, the anemia is characterized by macrocytosis and hyperchromia and is not affected by iron. In the clinical cases, we recently reported[16] that the anemia was usually normocytic, but may be hypochromic, and in such instances, the additional factor or an iron deficiency could not be excluded. Naturally, in such instances, the combination of iron and thyroid extract would accelerate the regeneration of erythrocytes and the formation of hemoglobin.

CONCLUSIONS

1. The daily administration of thyroid extract to adult, anemic, thyroidectomized rabbits caused a well-marked reticulocyte response, followed by a sharp and sustained rise in both hemoglobin and red blood cell counts. The hematopoietic effect was less with higher doses of thyroid. There was no significant effect on the blood of normal rabbits, during or after thyroid ingestion.

2. Intravenous liver extract had no effect on the blood of either the normal or thyroidectomized rabbits. Intramuscular liver extract caused a moderate reticulocyte response of both normal and thyroidectomized animals, and a slight but significant rise in the hemoglobin and erythrocyte counts of the latter group.

3. The administration of iron to normal and thyroidectomized animals had no effect on the reticulocytes of either group, and only a slight stimulative effect in the anemia of myxedema.

The authors wish to express their appreciation for the helpful suggestions of Drs. A. R. McIntyre and A. L. Bennet, and for the technical assistance of Dr. James Bena and Miss Mildred Braden.

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HYPERINSULINISM*

Report of a Second Case With Anatomic Findings

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RECORDED cases of proved adenomas of the pancreas are as yet uncommon; hence the following case report. In addition a few clinical studies and observations are recorded.

CLINICAL HISTORY

A white woman, aged thirty-eight years, was admitted to the Psychopathic Ward of the Los Angeles County General Hospital on June 17, 1932. On the previous day she had had a "nervous spell" and thrashed about. When the family doctor arrived, she answered questions in a confused manner, and then went into a deep sleep. During this attack she had an involuntary emptying of the bladder. When admitted to the hospital she was breathing heavily and throwing herself about the bed. Later she appeared entirely normal. The history was then obtained of three somewhat similar attacks, two years, six months, and two weeks earlier, respectively. These were "nervous hysterical spells" during which she became emotionally upset and was unable to control herself. Once while in this condition she beat her son unmercifully without rhyme or reason, another time she threw articles of furniture about. The outstanding feature during these attacks was the marked restlessness with the coarse jerking movements, throwing the body here and there, amounting at times to an acute maniacal state. This hypermotility would last a variable length of time up to two or three hours and afterward the patient would remain confused, though more quiet, for twenty-four hours. There was amnesia for the events of these periods.

The patient had been married six years and had one child who was living and well. She lived rather harmoniously with her husband and was described as an intelligent, industrious, and reserved personality. The past, family and systemic histories revealed no relevant material.

The patient was five feet eleven inches tall (the father was five feet eleven inches and the mother six feet in height). The heart rate was 104, temperature 97° F., and blood pressure 125/78. The teeth showed some caries and much repair work. The patient was obese, but the heart, lungs, abdomen, and extremities were negative. Also the neurologic examination and mental status were negative. Urinalysis and complete blood count were normal. Roentgenogram of the skull revealed no abnormality; that of the teeth showed several to be pulpless. The blood and spinal fluid Wassermann tests were normal; the colloidal benzoin test yielded 000003333300000.

No definite diagnosis was made except that a physical disorder was suspected. The patient was treated for epilepsy, and discharged to the parole department. She continued to have a repetition of her previous spells at more frequent intervals, so that she was readmitted to the psychopathic hospital three more times before the end of the year. On her third admission subsequent to an attack, the patient had difficulty in swallowing. She was roused from her semicomatose state by intravenous glucose, which the interne preferred to tube feeding. No special significance was attached to this experience until another admission, Dec. 20, 1932, when the interne recalled the previous improvement with glucose, and roused the patient

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to consciousness within five to ten minutes by the intravenous injection of 50 c.c. of a 50 per cent solution. A fasting blood sugar determination in the morning was 44 mg. per cent. It was now established that the attacks were due to a hypoglycemia of undetermined origin. An adenoma of the pancreas was the suspected anatomic lesion, since a diagnostic survey of the other endocrine organs was essentially negative.

Other laboratory tests included the following: icteric index, 10 units; blood cholesterol, 186 mg.; blood calcium 10.7 mg. The long bones were normal on x-ray.

TABLE I

GLUCOSE TOLERANCE TESTS

	BLOOD SUGAR MG. PER CENT					
	FASTING	½ HR.	1 HR.	2 HR.	3 HR.	4 HR.
January 3, 1933	44	106	113	96	69	
February 1, 1933	55		130	132	72	
February 27, 1935	53		141	139	120	61
	Fasting	*15'*	*30'*	*45'*	*60'*	
Adrenalin 5 minims by hypo	46	57	67	69	94	

Hypoglycemic attacks were becoming more numerous and so on June 8, 1933 an abdominal operation was performed by W. A. B. and the pancreas explored. No tumor was palpated and the patient was not subjected to a pancreatic resection. Since the latter procedure had failed to produce any satisfactory results in our first patient,[1] the surgeon decided to forego it.

The patient was placed on a high fat and low carbohydrate diet, but she had so many spells that this was discontinued. Attacks during the night in the last few months could be prevented if the patient had feedings at 1:00 A.M. and 5:00 A.M. The spells, however, became more numerous and the patient gained rapidly in weight. In the meantime our first patient had died, necropsy[2] revealing an adenoma in the pancreas which had not been located at two operations. On the basis of this experience the present patient was reoperated on March 21, 1935 by W. A. B. This time the spleen was removed and about two-thirds of the pancreas resected. The resected portions of the pancreas contained two adenomas, each about 1 cm. in diameter. Neither of these tumors was detected by palpation prior to the resection. The operation was rather prolonged (3½ hours) owing to previous adhesions and the patient died 32 hours later in respiratory failure, with temperature 107 degrees F., pulse 170 and B.P. 75/60.

SURGICAL PATHOLOGY—PANCREAS AND SPLEEN (DR. EDGAR MAUER)

Gross examination.—The specimen is a portion of pancreas and adherent fatty omentum weighing 45 gm. It consists of the tail and a small portion of the body of the pancreas measuring 5.5 by 3 by 1 cm. (Fig. 1). In the central portion of the body of the pancreas adjacent to the point of transection is a small, firm nodule completely surrounded by normal appearing pancreatic tissue and peritoneum. On section, this nodule is composed of soft gelatinous yellow tissue containing tiny white fibrous strands. No lobulations are made out. Just adherent to this nodule, directly beneath the splenic vein, is another small mass measuring 3 by 2 by 1 cm., even softer in consistency than the nodule and on section likewise presenting a gelatinous surface, darker yellow in color, with fine strands of fibrous tissue. The surface is smooth and homogeneous. Both nodules are fairly well demarcated from the surrounding pancreatic tissue. Adherent to the tail of the pancreas is a small mass of friable granulation tissue.

Accompanying the specimen are a cutaneous cicatrix, 22 cm. long, and the spleen 11 cm. long, 6 cm. wide and 3.5 cm. thick. The capsule of the spleen is smooth and slightly wrinkled. On section the parenchyma is firm. The Malpighian corpuscles are prominent. No gross pathologic changes are noted.

Microscopic Examination.—Pancreas: Sections through the tumor nodules, formalin fixed and stained with hematoxylin and eosin, show rather diffuse cellular masses, with a

very scanty stroma between the tumor cells. The tumor nuclei are round to oval and vary somewhat in size; the largest ones often have irregular contours and stain very darkly with hematoxylin; some nuclei are spindle-shaped. One to three nuclei are present in many of the cells. No mitoses are found. The pale, pink blue cytoplasm is scant and homogeneous. The supporting stroma is rich in capillaries; in a few places near the margin of the tumor nodule it forms broad bands. The fibrous connective tissue capsule, which partly surrounds the nodule, contains some isolated pancreatic acini and small ducts, but no tumor cells.

In another section of the pancreas (not through the tumor nodule) the majority of the islets are two to four times the normal size. These have cells with large, oval, blue-staining nuclei and also small cells with round, deeply staining (blue black) nuclei. In all of these, the cytoplasm is scant and pale pink blue. One enlarged islet has greatly distended capillaries. The pancreatic acini appear normal.

Spleen: The Malpighian corpuscles are numerous; many of the central arteries are thickened and have small lumina. The splenic pulp is very cellular; neutrophiles and lymphocytes are the predominating cells.

Fig. 1.—Gross specimen of pancreas and one tumor removed at surgery.

NECROPSY FINDINGS

The body was that of a white female, stated age forty-five years. There was a surgical incision in the abdomen; 2 cm. to the left of the umbilicus, about 20 cm. long, held together by skin clips, and containing two rubber drains in its middle portion. There was a 6 cm. incision extending laterally from the midportion of the large incision. There were eight needle punctures over the precordium. In the fossa of the right elbow was a 3 cm. skin incision, the edges approximated by a few silk sutures. To the right of this were several small needle punctures. The surrounding skin was blue red. There was about 3 cm. of subcutaneous fat in the abdomen and chest. The rubber drains and one gauze bandage were in the left upper part of the abdominal cavity. The greater part of the omentum lay in the left upper portion. There was no fat necrosis.

The spleen and the tail of the pancreas were absent. The soft tissue where the organs had been removed was red, and there was sanguinoturbid fluid in the upper peritoneal cavity. Flakes of fibrin were adherent to the stomach and the surfaces of the right and left lobes of the liver. There were fibrous adhesions about the gallbladder, the greater curvature of

the stomach, and between a few loops of the small intestine. The diaphragm was at the third interspace on the right, and at the fourth rib on the left.

The left lung lay freely in the pleural cavity, and the right was attached to the posterior chest wall by several fibrous stringlike adhesions. Both were collapsed after the removal of the breast plate. The right lung weighed 580 gm., the left 500 gm. Both were crepitant and a deep red. The tracheal and bronchial mucosa was dark red and the lumen contained a small amount of blood tinged mucus. The linings of the pulmonary artery and veins were grossly normal.

The pericardium was smooth and shining and the heart weighed 380 gm. There was a moderate increase in the subepicardial fat over the ventricles. Numerous dark red purple subepicardial petechial hemorrhages were noted posteriorly, at the base of the left ventricle. The coronary arteries were normal except for an occasional small fatty plaque. The measurements of the cardiac orifices were as follows: aortic, 7 cm.; pulmonic, 7.8 cm.; mitral, 9.4 cm.; tricuspid, 12.3 cm. The left ventricular wall at the base measured 1.5 cm.; at the apex, 1 cm. The right ventricular wall was 5 mm. thick. There was no gross scarring. The myocardium was red brown. The thoracic and abdominal aorta contained several bright yellow plaques.

The liver weighed 2,000 gm., was pale brown yellow and felt somewhat soft. The liver lobules were distinct. The gallbladder wall was fibrous. The lumen contained about 36 small faceted stones, 4 mm. in diameter, and some dark green bile. The cystic, hepatic, and common bile ducts were patent. The portal vein, hepatic artery, inferior vena cava, and head, and a small part of the body of the pancreas were present. The stump had one small white yellow region of fat necrosis 3 by 5 mm. The portion of the pancreas within the body was about 8 to 10 cm. long and 1.5 cm. wide. It was soft and there were no nodules either visible or palpable.

The adrenals were imbedded in a considerable amount of adipose tissue. Both were very soft. The cortex was narrow. The central portions were brown-orange. The kidneys together weighed 300 gm. and were pale red brown. The capsules stripped easily. The cortex averaged 8 mm., and in a few places showed dark red streaks which appeared to be small hemorrhages. The ureters were grossly normal.

The uterus was slightly enlarged because of several subserous and intramural white nodules 1 cm. in diameter. The cervical canal was moderately dilated, and in its wall were several small cysts.

The fallopian tubes were dark red. The ovaries were small and fibrous.

The brain weighed 1250 gm. After hardening in 10 per cent formalin, the brain looked somewhat small. Atrophy of the cerebral convolutions was found, most pronounced over the anterior portions of the hemispheres. The left hemisphere was somewhat larger than the right. The pia-arachnoid was opalescent and thickened. There were no signs of increased intracranial pressure. The vessels at the base of the brain showed no atheromatous change, in fact, the vessel walls were thin. Section through the tips of the temporal lobes revealed considerable enlargement of the left ventricle. There was an associated diminution in size of the basal ganglia on the left side. Sections of the remainder of the cerebrum showed no abnormalities. Section of the brain stem and cerebellum revealed no pathology.

Unfortunately, portions of the endocrines and most important organs removed for microscopic study were lost. Hematoxylin and eosin sections of the motor cortex, tuber cinereum, hippocampal gyrus, and cerebellum were normal.

The pathologist's diagnosis was adenoma of the islets of Langerhans with probable low-grade malignancy. The diagnosis of carcinoma was made on the infiltration of the capsule with tumor cells. There were no metastases to regional glands or elsewhere. The tendency to malignancy had been commented on by several authors reporting cases although the appearance of the tumors as a whole was that of adenoma. The operative finding of two adenomas of the islets was conclusive proof of the pancreatic origin of the hypoglycemia. The inability to detect these tumors by palpation was of interest. Unfortunately special stains to determine the type of islet cell involved were not obtained.

CHARACTER OF THE ATTACKS

Information concerning hypoglycemic attacks is obtained indirectly from reports by the patient and relatives and directly by observation. The latter determines the physical findings, and of course permits more accurate description of all the objective changes. Usually the number of spells seen by the physician is limited and once the diagnosis is established, the attacks are terminated early in their course, so that the completed, uninterrupted march of symptoms is interfered with. In the present case, on many occasions food was withheld for various purposes and the development of hypoglycemic symptoms noted under controlled conditions. Some of these test observations are presented in Table II. The hypoglycemia was always terminated before the patient lost consciousness or developed convulsions.

The hypoglycemic manifestations were varied and numerous. Convulsions were a frequent symptom though one never occurred under our direct observation. At least some of these were of the generalized type. The attacks differed from isolated convulsions inasmuch as restlessness, hypermotility and confusion usually preceded the seizures by a variable period up to several hours and again after the convulsion the patient was often confused for as long as twenty-four hours. For instance the nurse's notes would read: "5:20 A.M., convulsion. 8:00 A.M., in full restraint: sterterous breathing. 11:00 A.M., unable to swallow liquids. 2:00 P.M., involuntary urination." The patient was in a state of confusion with varying degrees of consciousness for the subsequent two days.

Although convulsions were frequently the outstanding hypoglycemic manifestation, this was not always the case. As a matter of fact, in the earliest attacks the mental aspects, e.g., confusion, irritability and maniacal outbursts, were responsible for her being committed to the psychopathic ward. At times the patient had attacks described by the mother as "hysterical," in which she walked about in a daze, only partially or intermittently responsive to conversation. At times she was facetious, coquettish, and euphoric and at other times appeared to have difficulty in finding the proper words to say, often evincing perseveration. Restlessness, grimacing movements and cessation of speech (apathy) were early manifestations of hypoglycemia.

Early symptoms in different tests on withholding food were hunger, headache, drowsiness, lightheadedness, ataxia, slowness in answering questions, disorientation, difficulty in performing simple mathematics, rolling of the eyes and increased mobility. Tachycardia, perspiration, tremor, and dilatation of the pupils were conspicuous by their absence. Subnormal temperature as low as 96° F., noted several times during the hypoglycemia, is a symptom which has not been adequately stressed in this condition. At times the patient presented a positive Babinski reflex, unilaterally or bilaterally. Once previous to the recognition of the clinical diagnosis the presence of Babinski reflexes with mental changes led to a suspicion of frontal lobe neoplasm.

A few clinical features of this case call for further comment. The slow development of symptoms is of interest. After an overnight fast of sixteen hours, although a low blood sugar was found, no symptoms would be apparent. At first twenty-three hours elapsed before symptoms appeared. This feature is

probably to be correlated with the relatively gradual development of the hypoglycemia. In other words, the lack of development of symptoms after an overnight fast does not exclude hypoglycemia or hyperinsulinism. Sometimes the blood sugar would be stationary at some hypoglycemic level and symptoms would appear only after this state had existed for one or more hours. For instance, on May 18, 1933, the patient had breakfast at 7:00 A.M. and was busily engaged in making beds in the ward. At 11:00 A.M. the blood sugar was 42 mg. per cent but we were unable to detect any symptoms. At 1:10 P.M. she became drowsy and went to bed. She showed a tendency to perseveration, could not recall the month or year and could not multiply 3×10. She could not be made to rise from the bed. The blood sugar test obtained at 1:30 P.M. was 41 mg. per cent. There was a marked symptomatic change, but no apparent change in the venous blood sugar.

TABLE II

EFFECTS OF WITHHOLDING FOOD

DATE 1933	TIME	BLOOD SUGAR MG. %	PULSE	SYMPTOMS
4/4	7:30 A.M. Breakfast			
	9:00 A.M.	110	100	None
	10:00 A.M.	103	96	None
	11:20 A.M.	88	98	None
	12:20 P.M.	62	96	None
	1:20 P.M.	60	88	None
	2:30 P.M.	54	88	Hungry, weak. Slight perspiration Headache
	3:30 P.M.	54	90	None
	4:30 P.M.	53	86	None
4/6	Midnight feeding			
	8:00 A.M.			Slightly weak and somewhat hungry
	9:00 A.M.	47		Hungry
	10:00 A.M.	48		None
	11:00 A.M.	51		Headache
	1:00 P.M.	45		Tremor of hands (slight). Slightly hungry
	2:00 P.M.	48	80	Slightly drowsy(?)
	3:30 P.M.	43	92	Occasional lightheadedness
	4:30 P.M.	45	90	Slightly hungry, mild headache, tired
4/13	7:00 P.M. Last feeding night before			
	9:00 A.M.	48	84	Slightly hungry
	1:00 P.M.	46	104	Slight headache
	2:00 P.M.	39	120	Coquettish, restless, threw arms over head, slid down in chair, wrapped coat about her aimlessly. "I'm acting foolish but I can't help it." Put arms about doctors familiarly. Regained self composure rapidly with glucose intravenously
4/14	6:00 P.M. Last feeding night before			
	8:15 A.M.	46		None
	12:00 Noon	52		None
	2:25 P.M.	55		Apathetic
	3:00 P.M.	54		Lying down with eyes closed. Stereotyped answers. "All right." "Sir." Very restless, thrashing around in bed, grimacing movements of face, making whining sounds, could read date in newspaper, but could not cooperate in most tests

The variability of the attacks is apparent from the foregoing discussion. The spells occurred most frequently after the longest interval without food, i.e. in the early morning. This was particularly so later in the course of the illness. The patient would be found in the morning in a disturbed state of consciousness by her mother, or would have convulsions before breakfast. Feedings during the night were necessary to control the symptoms.

A word as to the general importance of hypoglycemia to the neurologist may not be amiss. The observation of neurologic symptomatology on the basis of hypoglycemia has a profound significance for functional pathology of the nervous system. Transient neurologic manifestations have been observed by clinicians for years. Transient hemiplegias, for instance, have been the basis of much theoretical discussion and controversy for a long time. In hypoglycemia, where neurologic symptoms unfold with almost experimental exactitude as the blood sugar falls and then rapidly abate with carbohydrates, one has a clinical palpable mechanism, almost a laboratory experiment, conclusively demonstrating at least one organic basis for transient cerebral pathology. This clinical condition from a scientific point of view presents the best and most readily demonstrable evidence for transient changes of the type referred to.

SPECIAL STUDIES

1. *The Effect of Exercise on the Blood Sugar.*—Exercise or work is dependent on carbohydrate energy. Unless the glycogen stores are depleted no diminution in the blood sugar results. Hence moderate exercise in the normal person effects no change in the blood sugar. On the other hand, pronounced expenditure of energy for a short period raises the blood sugar, and prolonged exercise by depletion of reserve glycogen may lead to a hypoglycemia.[3] In the patient with clinical hypoglycemia, who presumably has only a small amount of carbohydrate reserve available, it was thought that exercise of a moderate degree might produce more marked changes in blood sugar than in the normal. This might reflect itself in the clinical symptoms as well as in the blood chemistry. In our first patient who had a pancreatic islet adenoma there was the history of being obliged to give up football because of early resulting fatigue and dizziness. These symptoms were present 18 months before he came to medical care. Later he attributed a few convulsions to overexertion. Both the present patient and still another whom we have under observation complained of inability to carry out their usual work or any increased physical demands without undue ready exhaustion and other hypoglycemic symptoms. Hence a few objective tests were performed.

The effect of exercise was studied in 14 different trials. The first 7 tests were discarded because the test periods were short and the absence of differences from control periods indicated that the experimental setup was inadequate for demonstrating any change, if such existed. In the last 7 experiments (Table III) the patient was given breakfast at 7:00 A.M. and no food thereafter. Blood sugar tests were taken at 11:00 A.M., 1:30 P.M. and 4:30 P.M. In four instances the patient was kept at work making beds and helping about the ward and in three instances she was at rest in bed. In all except two tests hypoglycemic symptoms appeared before 4:30 P.M., and orange juice was administered early.

The two exceptions were instances in which the patient was confined to bed. The average 11 o'clock blood sugar in the control groups was 55 mg. per cent and in the exercise group 47 mg.; the 1:30 P.M. reading was 46 mg. in the control and 40 mg. in the exercise group. Although these tests suggest that exercise does lower the blood sugar level and hasten the appearance of hypoglycemic

TABLE III

EFFECT OF EXERCISE ON HYPOGLYCEMIA

DATE	STATE OF ACTIVITY	TIME	BLOOD SUGAR LEVEL	SYMPTOMS
5/18/33	Ward work	11:00 A.M.	42 mg.	None
		1:10 P.M.		Drowsy, lying down. Tendency to perseveration, inability to recall month and year, could not multiply 3 × 10, could not rise up in bed
		1:30 P.M.	41 mg.	
5/19/33	Bed rest	11:00 A.M.	58 mg.	None
		1:30 P.M	50 mg.	None
		4:30 P.M.	53 mg.	None
5/22/33	Ward work	11:00 A.M.	46 mg.	None
		1:30 P.M.	37 mg.	Shortly before 1:30 P.M., patient began to stagger and on being put to bed fell asleep
5/23/33	Bed rest	11:00 A.M.	60 mg.	None
		12:00 Noon		None
		1:30 P.M.	46 mg.	
		2:30 P.M.	45 mg.	Restless and facetious, given orange juice
5/29/33	Ward work	11:00 A.M.	51 mg.	None
		1:30 P.M.	41 mg.	Acting queerly, orange juice given
5/31/33	Bed rest	11:00 A.M.	46 mg.	None
		1:30 P.M.	42 mg.	Restless, eyes rolled about, voice did not seem natural. Given orange juice with relief of symptoms
6/2/33	Ward work	11:00 A.M.	47 mg.	None
		11:40 A.M.		Weak, went to bed
		12:20 P.M.	41 mg.	Slow in answers, tho correct. Pulse 90, no tremor, no perspiration, no dilatation of pupils, 9 × 7 equals 63

symptoms, the number of trials is small and the differences between the experimental and control groups are not very great.

2. *Spinal Fluid Pressure and Sugar Determinations.*—The relation between the levels of spinal fluid sugar and blood sugar is an important and interesting one. It may have some bearing on the symptoms of hypoglycemia and is significantly related to the spinal fluid pressure. Table IV records an unusual observation, namely, a greater quantity of glucose in the spinal fluid than in the blood.

3. *Effect of Insulin on the Sugar Utilization Rate.*—Dr. Douglas Drury and Dr. Paul Greeley of the Physiology Department have been conducting tests in the laboratory on carbohydrate utilization as affected by various endocrine

changes such as hypophysectomy, pancreatectomy and insulin injections. They suggested a study of the insulin response on glucose consumption in hyperinsulinism, and hence performed the following test, the results of which are published through their courtesy.

TABLE IV

RELATION OF BLOOD SUGAR TO SPINAL FLUID PRESSURE

TIME	BLOOD SUGAR MG. %	SPINAL FLUID SUGAR MG. %	SPINAL FLUID PRESSURE MM. H_2O	SYMPTOMS
8:40 A.M.	54	83	165-208*	None
12:46 P.M.	42	33	120	Slightly euphoric. Retarded. Difficulty in finding proper words, difficulty in reading. Some perseveration. Can perform simple mathematics. No tremor, tachycardia, restlessness or dilated pupils
3:00 P.M.	50	31	40	Very drowsy. Answered questions with eyes closed. Still oriented. Perseveration marked. Few mistakes in simple multiplication. Pulse 72. No tremor or restlessness.

The spinal needle was withdrawn after each puncture, so that the low spinal fluid pressures after the initial test are probably due to the withdrawal and subsequent leakage of spinal fluid. For further comments on these findings the reader is referred to a previous report.[2]

*The pressure oscillated for more than ten minutes; a constant reading could not be obtained.

TABLE V

DEXTROSE UTILIZATION

INJECTION TIME FROM	TO	GRAMS GLUCOSE PER HOUR	MG. GLUCOSE PER KG. BODY WT. PER HOUR	BLOOD SUGAR MG. %
9:40				66
10:00	10:52	?	?	111†
10:52	11:52	11.76	130	80
11:52	12:52	15.4	170	90
12:52	1:44	14.1	155	87
1:44	2:27*	22.0	242	74
2:27	2:57	30.4	335	65
2:57	3:30	36.0	395	74
3:30	4:04	35.0	385	87

*20 units of insulin given at 2:02 P.M.

†All blood sugar determinations were obtained at the end of injection intervals.

Glucose was injected intravenously from a burette in quantity sufficient to maintain the blood sugar at a constant normal level (Table V). For a blood sugar concentration between 90 and 87 mg. per cent in this patient an injection rate of 155 mg. per kilogram body weight per hour was required. The injection of 20 units of insulin intravenously increased the utilization rate markedly to 385 mg. of glucose per kilogram body weight per hour. Apparently insulin acted in the same direction in this patient as it does in the normal. Quantitative comparisons cannot be made since the average normal response in human beings is unknown.

CONCLUSIONS

A case of hyperinsulinism due to pancreatic islet cell adenomas is presented. It is of interest in that the adenomas could not be palpated even at operation. The relatively long latent period after withholding of food before symptoms developed warrants comment and bespeaks the relatively mild chronic degree of hypoglycemia present at the outset.

Prolonged exercise lowered the blood sugar in this patient as compared to periods of rest.

The spinal fluid pressure was elevated slightly above normal. At this point the spinal fluid sugar was higher than the blood sugar.

The glucose utilization rate in this patient was such that 1.5 gram of dextrose per kilogram body weight per hour was required to maintain a constant blood sugar level of 90 mg. per cent.

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CONTROL OF INTRACTABLE CLINICAL AND OF TOTAL EXPERIMENTAL DIABETES WITH PROTAMINE INSULIN*

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THE discovery by Hagedorn and his associates[1] that the precipitate produced by the combination of protamine with insulin provides a prolonged hypoglycemic effect, has made possible a great advance in the treatment of diabetes. Almost all of the numerous clinical reports that have appeared are highly favorable.[2]

Patients with severe diabetes treated for years with regular insulin have been found difficult to adjust with protamine insulin, but most observers believe that a satisfactory balance can usually be obtained. That exceptions occur is conceded by Wilder[2] who emphasizes the fact that the unstable diabetic remains unstable even when the new preparation is used.

The present report is concerned with six patients who had severe diabetes intractable to treatment with regular insulin; they comprise a group found most difficult to manage in a series of 400 diabetics. In addition, observations in five depancreatized dogs are presented. The use of protamine insulin in experimental diabetes revealed problems similar to those encountered clinically.

A. SEVERE CLINICAL DIABETES

These six patients had used insulin for periods of one to six years without achieving satisfactory management. During this time they were active and in fair health. The diabetes was intractable in the sense that it was found impossible to maintain aglycosuria by any variations in the size, number or timing of insulin injections that were attempted (Group I), or it was inexpedient to do so because of frequent reactions (Group II). The three patients in the first group had persistent, nocturnal hyperglycemia and regularly spilled sugar before and after breakfast. Three patients of the second group were unstable; they exhibited marked variations in blood sugar levels and had frequent reactions.

The patients were hospitalized, diurnal blood sugar curves were recorded on their regular insulin regimens, and treatment with protamine insulin started. They subsequently reported at weekly intervals for examination of a twenty-four-hour urine specimen and for a fasting blood sugar determination. Daily, fractional examinations of the urine were made and recorded by the patients, themselves. Protamine insulin was, at first, substituted for the evening dose of regular insulin, without marked effect. Later, by means of larger doses, changes

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For the privilege of obtaining regular supplies of protamine insulin we are indebted to Eli Lilly and Company, Indianapolis, Indiana.

in the time of administration, and more effective preparations containing traces of calcium or zinc, more decisive results were obtained. Protamine insulin has been continued for nine to eleven months.

CASE REPORTS

CASE 1.—Billy A., aged fifteen years; high school student. (Table I.) Diabetes was discovered at ten years of age. In April, 1935, when he came to the clinic after hospital observation and instruction, the urine was loaded with sugar in spite of insulin totalling 115 units, administered before meals at 8:00 A.M., 4:00 P.M., and midnight. For a year the fasting blood sugar determinations made were consistently high and twenty-four-hour urines examined contained 40 to 60 gm. of sugar with a diet containing 138 to 158 gm. of available glucose.

Protamine insulin was started before the evening meal on April 2, 1936, and proved more effective than the same number of regular insulin units. Aglycosuria was maintained after a few weeks with a smaller total-insulin dosage. Injections of the new preparation were then shifted to the morning at the same time as those of regular insulin. The carbohydrate in the diet was gradually increased from 100 to 150 gm. and the protamine dosage decreased from 50 to 30 units. The use of accessory insulin before breakfast has been continued. Aglycosuria has been maintained for eight months. Despite some low fasting blood sugar levels, there have been no clinical manifestations of hypoglycemia, perhaps due to the fact that food is taken at bedtime.

Summary.—With protamine insulin (present dosage 30 units) and accessory insulin (20 units), aglycosuria has been maintained for eight months, whereas for a year with regular

TABLE I

CASE 1

DATE	INSULIN	FASTING B.S.	URINE	DIET CH P F	WEIGHT	REACTIONS
4/15/35	60–15–40	–	65	100–80–120	118	+
7/ 1/35	60–15–40	303	+	80–80–120	118	+
4/ 2/36	60– 5–30	422	64	80–80–120	122	0
4/18/36	50–5–thirty	465	+	100–80–150	–	0
4/25/36	50–5–thirty	350	+	100–80–150	–	0
5/ 2/36	50–5–thirty	312	+	100–80–150	–	0
5/17/36	50–5–thirty	235	11	100–80–150	–	0
6/ 6/36	50–0–thirty-five	231	4	100–80–150	132	0
6/20/36	50–0–thirty-five	58	0	100–80–150	–	0
6/27/36	50–0–thirty-five	40	0	116–86–150	–	0
7/ 7/36	fifty 20–0–0 (Ca)	80	0	116–86–150	–	0
7/20/36	fifty 20–0–0 (Ca)	73	0	116–86–150	–	0
7/27/36	fifty 20–0–0 (Ca)	84	0	116–86–150	–	0
8/ 5/36	fifty 20–0–0 (Ca)	32	0	116–86–150	–	+
8/10/36	fifty 20–0–0 (Zn)	67	0	116–86–150	–	0
8/17/36	forty 20–0–0	186	0	116–86–150	–	0
8/24/36	forty 20–0–0	–	0	116–86–150	–	0
9/10/36	forty 20–0–0	105	0	116–86–150	–	0
10/ 5/36	forty 20–0–0	67	6	116–86–150	–	0
10/19/36	forty 20–0–0	71	0	116–86–150	–	0
10/31/36	forty 20–0–0	90	–	116–86–150	–	0
11/14/36	forty 20–0–0	74	–	116–86–150	–	0
11/23/36	forty 20–0–0	54	0	116–86–150	–	0
12/ 5/36	forty 20–0–0	133	0	116–86–150	–	0
12/14/36	forty 20–0–0	62	0	116–86–150	134	0
12/21/36	forty 20–0–0	56	0	116–86–150	–	0
1/ 9/37	forty 20–0–0	76	0	130–80–145	–	0
1/23/37	forty 20–0–0	64	0	130–80–145	–	0
2/ 6/37	forty 20–0–0	80	0	150–80–135	–	0
2/20/37	forty 20–0–0	74	0	150–80–135	–	0
3/ 6/37	thirty 20–0–0	84	0	150–80–135	–	0

insulin totalling 95 to 115 units, satisfactory control was impossible. The carbohydrate in the diet has been increased from 100 to 150 gm. This increase has seemed advisable in order to avoid reactions.

CASE 2.—Michael G., aged seventeen years, high school student. (Table II.) This patient was under our care at nine years of age when mild diabetes was discovered. After two years the regimen which he had been taught was gradually relaxed. He reported to the Clinic in April, 1932, with a severe diabetes characterized by persistent, nocturnal hyperglycemia. Reactions were frequent and made him appear dull and uncooperative. For four years his diabetes remained poorly controlled. A diurnal blood sugar curve was almost identical with that of Case I.

TABLE II

CASE 2

DATE	INSULIN	FASTING B.S.	URINE	DIET CH P F	WEIGHT	REACTIONS
4/24/32	40–0–30	278	20	75–60–155	78	+
12/18/33	60–0–15	444	+	75–60–155	83	+
1/22/34	30–0–20	445	80	75–60–155	85	+
6/25/34	30–0–20	400	60	70–70–160	93	+
6/21/35	60–0–20	410	+	70–70–160	105	+
3/26/36	50–0–20	388	+	70–70–160	108	+
4/11/36	50–0–twenty	112	+	100–75–130	112	+
4/18/36	30–0–twenty	–	40	100–75–150	–	0
5/18/36	30–0–twenty	–	44	100–75–150	–	0
5/23/36	30–0–thirty	294	60	100–75–150	–	0
6/ 6/36	30–0–forty	84	+	120–77–150	–	+
6/13/36	40–0–forty	80	+	120–77–150	–	0
6/25/36	40–0–forty	107	5	120–77–150	–	0
6/29/36	40–0–forty	82	26	120–77–150	–	0
7/27/36	forty–0–0 (Zn)	41	13	120–77–150	–	0
8/ 5/36	thirty 10–0–0	42	15	120–77–150	–	0
8/17/36	thirty 10–0–0	123	10	120–77–150	118	0
8/24/36	thirty 10–0–0	70	0	120–77–150	–	0
8/31/36	thirty 10–0–0	73	3	120–77–150	121	0
9/ 3/36	thirty 10–0–0	151	5	120–77–150	–	0
9/ 9/36	thirty 10–0–0	155	0	120–77–150	–	0
9/21/36	thirty 10–0–0	94	0	120–77–150	–	0
10/ 5/36	thirty 10–0–0	129	4	120–77–150	122	0
10/19/36	thirty-five 0–0	46	0	120–77–150	–	0
10/24/36	thirty-five 0–0	145	–	120–77–150	–	0
10/31/36	thirty-five 0–0	174	–	120–77–150	–	0
11/ 7/36	thirty-five 0–0	140	0	120–77–150	–	0
11/14/36	thirty-five 0–0	55	0	120–77–150	123	0
11/21/36	thirty-five 0–0	44	0	120–77–150	–	0
11/28/36	thirty-five 0–0	46	3.8	120–77–150	–	0
12/ 5/36	thirty-five 0–0	47	0	120–77–150	–	0
12/12/36	thirty 0–0	60	0	120–77–150	–	0
1/ 9/37	thirty 0–0	80	0	140–76–140	–	0

Protamine insulin was started on March 26, 1936. The evening dose of 20 units insulin was replaced with 20 units protamine insulin, and the morning injection of 50 units insulin was continued. After several shifts in dosage, improvement followed the use of 40 units of insulin before breakfast and 40 protamine insulin before supper. Protamine insulin containing zinc was started on July 6; this was given in the morning with the regular insulin. For six months he has been almost constantly sugar-free. The carbohydrate in the diet has been substantially increased and a gain in weight of 15 pounds has resulted. The protamine insulin dosage has been reduced to 30 units daily and the use of accessory insulin discontinued.

Summary.—Insulin, 70 units daily, failed to prevent glycosuria before and after breakfast and caused frequent reactions. The lack of stabilization continued for years. With protamine insulin, satisfactory control has been maintained for six months; the present dosage

is 30 units and regular insulin is no longer required. A diet increase, including a feeding at bedtime, has seemed necessary to avoid reactions. No clinical manifestations have accompanied the frequent low fasting blood sugar levels.

CASE 3.—Mr. T. S., aged thirty-one years; electrical worker. (Table III.) Symptoms were first noticed nine years ago at the age of twenty-two years. It is of interest that a daughter became diabetic at the age of nine. Insulin, 50 to 100 units, was used for over six years but the patient always found the fasting and after breakfast urines loaded with sugar. A diurnal blood sugar curve was similar to those of Cases 1 and 2.

The use of protamine insulin was begun on April 7, 1936, with almost immediate benefit. Preparations containing calcium were given from July 7 to October 9; since then preparations with zinc have been used. Protamine insulin, 50 units before breakfast, and insulin, 30 units before breakfast, finally maintained aglycosuria. A reduction to 40 protamine insulin and 10 units insulin before breakfast was made, and recently the accessory insulin has been omitted.

Summary.—With the use of protamine insulin, satisfactory management has been maintained for a period of eight months. This is now possible with a single morning injection of 40 units protamine insulin, as contrasted with the failure during six years to maintain aglycosuria on a more restricted diet with regular insulin in amounts of 50 to 100 units daily.

TABLE III

CASE 3

DATE	INSULIN	FASTING B.S.	URINE	DIET CH P F	WEIGHT	REACTIONS
7/21/30	35–0–30	444	+	75–75–154	164	0
3/30/31	45–20–35	384	+	80–70–121	164	+
3/12/36	40–0–40	450	64	80–70–121	–	0
3/28/36	20–10–20	392	+	80–70–121	150	0
4/ 6/36	30–0–20	400	38	100–65–100	–	0
4/18/36	20–0–twenty	289	+	110–70–150	–	0
4/25/36	25–0–twenty-five	209	0	110–70–150	–	0
5/ 9/36	25–0–twenty-five	137	20	110–70–150	148	0
5/16/36	25–0–thirty	116	–	110–70–150	–	0
5/23/36	25–0–thirty	157	–	110–70–150	–	0
6/ 5/36	25–0–thirty	133	8	130–76–150	153	0
6/26/36	25–0–thirty	72	6	130–76–150	–	0
7/13/36	fifty 0–0 (Ca)	40	0	130–76–150	–	0
7/20/36	fifty 0–0 (Ca)	58	8	130–76–150	–	0
8/14/36	fifty 10–0–0	118	0	130–76–150	–	0
8/21/36	fifty 10–0–0	157	0	130–76–150	–	0
9/11/36	fifty 10–0–0	174	–	130–76–150	–	0
9/18/36	fifty 20–0–0	72	0	130–76–150	–	0
10/ 9/36	fifty 20–0–0	42	–	130–76–150	–	0
10/19/36	fifty 20–0–0 (Zn)	579	+	130–76–150	–	0
10/24/36	fifty 20–0–0	42	–	130–76–150	–	+
10/31/36	fifty 10–0–0	45	–	130–76–150	–	0
11/14/36	fifty 10–0–0	68	–	130–76–150	–	0
11/23/36	fifty 10–0–0	53	–	130–76–150	–	0
11/28/36	forty 10–0–0	176	–	130–76–150	–	0
12/ 4/36	forty 10–0–0	148	0	130–76–150	153	0
12/11/36	forty 10–0–0	176	0	130–76–150	–	0
12/18/36	forty 10–0–0	73	0	130–76–150	–	0
1/ 9/37	forty 10–0–0	64	0	150–78–140	–	0
1/30/37	forty 0–0	78	0	150–78–140	–	0
2/15/37	forty 0–0	74	0	150–78–140	–	0

CASE 4.—Mrs. V. B., aged thirty-nine years, office worker. (Table IV.) Diabetes was recognized at twenty years of age. At twenty-eight years a toxemia of pregnancy in the last trimester caused unconsciousness and convulsions and was treated by abortion. When first examined by us in April, 1929, hyperthyroidism was evident and a thyroidectomy was per-

formed. Following the operation the diabetes was easily controlled by small doses of insulin until 1932, when she lost her job and neglected to follow her diet. When she came to the Clinic, 50 to 60 units of insulin daily produced reactions but did not control the diabetes.

After seven months of difficulty in management, protamine insulin was started on May 2, 1936. For six weeks no improvement was noted due to recurrent fever finally diagnosed as subsiding undulant fever. Fairly satisfactory control was then effected. The total-insulin dosage has been gradually reduced because of occasional reactions until she now receives only 20 protamine insulin before breakfast. The diet has not been increased because of overweight.

Summary.—Satisfactory control has been effected but the diabetes remains slightly unstable. Protamine insulin has permitted a progressive reduction in total-insulin units. Accessory insulin, at first required, was later omitted because of occasional reactions before the noon meal.

TABLE IV

CASE 4

DATE	INSULIN	FASTING B.S.	URINE	DIET CH P F	WEIGHT	REACTIONS
11/ 1/35	25–20–15	300	20	110–60–130	146	++
3/14/36	30–0–20	320	10	100–60–125	142	++
5/ 2/36	30–0–twenty-five	308	30	100–63–112	–	0
5/25/36	30–0–thirty	286	20	100–63–112	140	0
6/ 4/36	30–0–thirty	286	39	100–63–112	–	0
6/22/36	fifty 20–0–0 (Ca)	244	6	100–63–112	–	0
7/ 6/36	fifty 20–0–0 (Ca)	100	3	100–63–112	–	0
7/13/36	fifty 10-0-0	172	6	100–63–112	–	0
7/20/36	fifty 0–0	55	0	100–63–112	–	+
7/27/36	forty 0–0	81	11	100–63–112	–	0
8/10/36	forty 0–0	88	26	100–63–112	–	0
8/17/36	thirty-five 10–0–0	75	0	100–63–112	143	0
8/31/36	thirty 10–0–0	94	0	100–63–112	–	0
9/10/36	thirty 10–0–0	73	4	100–63–112	–	0
10/ 9/36	thirty 10–0–0 (Zn)	183	–	100–63–112	–	0
10/26/36	thirty 10–0–0	64	4	100–63–112	–	+
11/14/36	twenty 10–0–0	204	+	100–63–112	–	0
12/11/36	twenty 10–0–0	72	0	100–63–112	–	+
12/18/36	twenty 0–0	100	3	100–63–112	–	0

CASE 5.—Miss H. K., aged twenty-five years. (Table V.) Diabetes was discovered in 1930, and treatment with insulin started. She came to the Clinic in March, 1932, with the diabetes uncontrolled. Weight 114 pounds, height 65 inches. Under our observation of almost four years she was in fairly good general health, but the urine could not be kept consistently sugar-free. Insulin in doses of 30-0-20 produced frequent slight reactions.

The use of protamine insulin was begun in May, 1936; since July sixth preparations containing zinc have been employed. Fifty units of protamine insulin before breakfast permitted a urinary sugar output of 5 to 40 gm. The supplemental use of 15 units of regular insulin before breakfast reduced the amount of glucose excreted. Improvement has been progressive and satisfactory control has recently been established. Reactions no longer occur, due in part to an increased diet with a feeding at bedtime.

Summary.—With regular insulin it had been impossible for six years to give amounts adequate to control the diabetes because of reactions. Protamine insulin and accessory insulin have made possible progressively improved control. The diabetes remains slightly unstable.

CASE 6.—Mrs. D. T., aged thirty years. (Table VI.) Mild diabetes was detected five years ago, shortly after the appearance of symptoms, and insulin treatment started. The patient was undernourished and of nervous temperament. In 1932, about five hours after 15 units of insulin and breakfast, she lost consciousness and suffered a laceration of the scalp. During four years of observation she remained well and active but had marked variations

TABLE V

CASE 5

DATE	INSULIN	FASTING B.S.	URINE	DIET CH P F	WEIGHT	REACTIONS
10/ 8/34	30–0–10	258	30	110–95–190	118	0
5/13/35	30–0–25	220	18	120–65–190	110	+
4/29/36	30–0–20	286	32	130–60–180	113	+
5/ 7/36	30–0–twenty	167	18	130–60–180	112	0
5/18/36	30–0–twenty	231	6	130–60–180	–	0
5/25/36	30–0–twenty	119	36	130–60–180	–	0
6/ 1/36	25–0–thirty	191	–	130–60–180	–	0
6/19/36	fifty 15–0–0	105	0	140–60–170	114	0
6/25/36	fifty 15–0–0	70	0	140–60–170	–	0
6/29/36	fifty 15–0–0	82	0	140–60–170	–	0
7/ 6/36	fifty 0–0	115	11	140–60–170	–	0
7/13/36	fifty 0–0 (Zn)	105	40	140–60–170	–	0
7/20/36	fifty 0–0	105	22	140–60–170	–	0
7/27/36	fifty 0–0	70	5	140–60–170	–	0
8/ 3/36	fifty 0–0	56	23	140–60–170	–	0
8/17/36	forty 10–0–0	122	28	140–60–170	–	0
8/24/36	forty 10–0–0	71	10	140–60–170	–	0
8/31/36	forty 10–0–0	143	24	140–60–170	114	0
9/10/36	forty 10–0–0	218	11	140–60–170	–	0
10/12/36	forty 10–0–0	112	5	140–60–170	–	0
10/19/36	fifty 15–0–0	123	0	140–60–170	–	0
11/ 2/36	fifty 15–0–0	124	15	140–60–170	–	0
11/ 9/36	fifty 10–0–0	94	0	151–62–171	–	0
11/23/36	fifty 10–0–0	163	7	151–62–171	117	0
11/30/36	fifty 10–0–0	75	5	151–62–171	118	0
12/14/36	fifty 15–0–0	116	0	151–62–171	–	0
12/28/36	fifty 15–0–0	80	0	151–62–171	–	0
1/ 9/37	forty 15–0–0	76	0	151–62–171	120	0
1/30/37	forty 15–0–0	80	3	151–62–171	–	0
2/15/37	forty 15–0–0	82	2	151–62–171	122	0

TABLE VI

CASE 6

DATE	INSULIN	FASTING B.S.	URINE	DIET CH P F	WEIGHT	REACTIONS
10/31/31	15–0–0	222	+	80–55–130	118	0
9/21/32	15–0–0	164	+	90–70–150	–	++++
11/17/33	15–0–0	310	+	90–70–150	119	0
11/ 6/34	15–0–0	272	+	90–70–150	–	0
3/16/36	20–0–15	205	+	120–60–130	–	+
3/26/36	15–0–fifteen	173	+	120–60–178	119	0
4/ 4/36	15–0–fifteen	250	+	120–60–178	–	0
4/24/36	15–0–fifteen	188	1.3	120–60–178	–	0
5/13/36	15–0–fifteen	100	0	120–60–178	–	0
6/ 1/36	thirty 0–0	132	0	140–60–170	119	0
6/ 9/36	thirty 0–0	–	75	140–60–170	–	0
6/23/36	thirty 0–0	370	+	140–60–170	–	0
7/ 2/36	thirty 10–0–0	192	20	140–60–170	–	++++
7/10/36	thirty 10–0–0 (Ca)	115	0	140–60–170	–	0
7/23/36	thirty 10–0–0	117	53	140–60–170	–	++++
8/ 7/36	10–0–thirty	187	+	140–60–170	119	0
8/18/36	10–0–twenty	217	+	156–66–177	–	0
8/28/36	10–0–twenty	146	+	156–66–177	–	0
9/ 4/36	10–0–twenty	225	+	156–66–177	–	0
9/25/36	10–0–twenty	143	–	156–66–177	–	0
9/29/36	twenty 0–0	216	42	156–66–177	–	0
10/ 9/36	twenty 0–0	167	7	156–66–177	–	0
11/ 5/36	twenty 0–0	227	–	156–66–177	–	0
11/20/36	twenty-five 0–0 (Zn)	120	31	156–66–177	122	0
11/25/36	twenty 10–0–0	100	0	156–66–177	–	+
12/14/36	twenty 0–0	130	6	156–66–177	–	0

in the fasting blood sugar level and in the urinary sugar output. Emotional disturbances produced hyperglycemia and unusual physical exertion, hypoglycemia.

Protamine insulin has been used since March 17, 1936. After worry over illness in her family on June 8, the twenty-four-hour urine contained 75 gm. of sugar. On July 1 and 22 she lost consciousness before breakfast and was revived by means of injections of epinephrine and subsequent feeding. The reactions were ascribed to the fact that she slept two or three hours later than usual. She now takes 20 units of protamine insulin before breakfast and remains unstable. Since her diet has been divided into five feedings, reactions have been avoided.

Summary.—This patient has very unstable blood sugar levels both with insulin and protamine insulin treatment. Severe reactions are apt to follow small doses. With the new preparation, alone, reactions have been prevented, in part by a feeding at bedtime. The carbohydrate allowance has been increased without causing increased glycosuria. Subjectively, great improvement is claimed. She remains an unstable diabetic and no effort is made to keep the urine consistently sugar-free.

DISCUSSION

The six cases reported had been treated with regular insulin for one to six years without achieving satisfactory stabilization. In one group (Cases 1, 2, and 3), successful management was prevented because of uncontrolled hyperglycemia and glycosuria during the early morning hours. By means of protamine insulin, alone in two instances, and with accessory insulin in one, it has been possible to maintain aglycosuria for six to eight months with fewer total-insulin units. Reactions have not occurred. Improvement has been progressive. An increase in the previously low carbohydrate allowance of the diet seemed not only advisable but actually necessary. That patients with diabetes of this type, described as "insulin wasters," can be stabilized with protamine insulin was suggested by the observations of Richardson and Bowie.[3]

In another group characterized by widely fluctuating blood sugar levels and by frequent reactions when regular insulin was given, less striking results were obtained. With the new preparation and a more liberal diet including additional feedings, reactions are more easily prevented and subjective improvement occurs. In two cases fairly satisfactory control has been effected but intermittent glycosuria, usually slight, persists. One of these patients requires accessory insulin. One very unstable diabetic remains very unstable but is subjectively better on an increased diet and avoids reactions. Efforts to maintain the urine sugar-free have been abandoned.

The six cases reported comprise a group which had proved the most difficult to treat satisfactorily with regular insulin in a series of 400 diabetics. Our observations support the clinical conclusion of other writers that severe diabetics are benefited by protamine insulin. The type of case characterized by persistently high fasting blood sugars and morning glycosuria is apparently readily controlled with the new preparation. Unstable diabetics are more effectively managed but remain unstable; in the exceptionally severe cases the improvement seems largely subjective.

B. EXPERIMENTAL DIABETES

Diabetes was produced in five dogs by depancreatization. Three of these animals had been maintained with regular insulin for approximately six months

prior to the present study. Two dogs were depancreatized and placed on protamine insulin immediately after the operation.

All of the animals were fed a routine diabetic diet consisting of carbohydrates 100, proteins 100, fats 90, which included 200 gm. of raw ground pancreas daily. The ration was ordinarily divided into two equal portions and fed at 8:00 A.M. and 5:00 P.M. Twenty-four hour urines preserved with toluol were examined quantitatively for sugar. These animals were not catheterized; because of variations in the daily output of as much as 100 per cent, all values recorded for urinary sugar represent the average five day excretion.

CASE REPORTS

Dog 1.—(Airedale 13.) Depancreatized Nov. 7, 1935, during last trimester of pregnancy. Insulin dosage after complete recovery from lactation was 14-0-14. Protamine insulin started July 29, 1936. Weight before operation 14.5 kg., present weight 15.4 kg.

Summary.—This dog was maintained on regular insulin for 8 months following pancreatectomy before being placed on protamine insulin. Protamine insulin with accessory regular insulin was given in the morning with the full day's ration. This controlled the hyperglycemia better than the regular insulin alone; the average glycosuria was controlled 50 per cent better. However, when daily rations were divided into two portions and 6 units of insulin and 16 units of protamine insulin were given in the morning, both the fasting and the diurnal blood sugar values were lower and more constant than when insulin alone was used.

TABLE VII

DOG 1

OBSERVATION	URINE SUGAR RANGE GM. – 24 HOURS	FASTING BLOOD SUGAR RANGE; MG. PER 100 C.C.	DIURNAL BLOOD SUGARS*
20 days; feedings 8 A.M. & 5 P.M. Insulin 14 – 0 – 14	6.6 – 11.9	222 – 300	222 – 66 – 200
20 days; single feeding, 8 A.M. Prot. Ins. 16 and Insulin 6 in A.M.	0 – 10.2	140 – 173	140 – 135 – 46
20 days; feedings 8 A.M. & 5 P.M. Prot. Ins. 16 before bkfst., Insulin 6 before supper	3.2 – 6.2	101 – 218	101 – 177 – 58

*Diurnal blood sugars determined at fasting, one hour after 8:00 A.M. meal, and nine hours after A.M. meal (or before supper).

Dog 2.—(Bull 18.) Depancreatized Dec. 20, 1935, during first trimester of pregnancy. Insulin dosage after recovery from pregnancy was 10-0-10, with feedings at 8 A.M. and 5:00 P.M. Protamine insulin started July 29, 1936. Weight before operation 20 kg., present weight 15.5 kg.

Summary.—This animal has been entirely sugar-free for three months on insulin and protamine insulin. The fasting blood sugar remains around 120 mg. Although the glycosuria and hyperglycemia were never very marked on regular insulin, it has been easier to prevent glycosuria by means of protamine insulin and accessory regular insulin.

Dog 3.—(Crossbreed Male R.) Depancreatized Aug. 18, 1936. Protamine insulin, 10 units, given on second postoperative day, when he was eating about one-third of the total diet. Mild distemper on sixth postoperative day. Protamine increased to 12 units; severe convulsion at 4:00 P.M., relieved by 20 gm. of glucose intravenously. From the eighth to the fifteenth day postoperative, ate regularly; had distemper and a stitch abscess.

From sixteenth to thirtieth day received 12 units of protamine insulin and full diet; had diarrhea twice and was sluggish occasionally during late afternoon. Urine sugar ranged from 0 to 3.9 gm. in twenty-four hours, and fasting blood sugar from 152 to 180 mg. Diurnal blood sugar readings—152, 115, 21 mg.

On thirtieth day slight convulsion at 4:00 P.M., on thirty-first day severe convulsion, stopped by food.

TABLE VIII

DOG 2

OBSERVATION	URINE SUGAR RANGE GM. – 24 HOURS	FASTING BLOOD SUGAR RANGE; MG. PER 100 C.C.	DIURNAL BLOOD SUGARS*
20 days; feedings 8 A.M. & 5 P.M. Insulin 10 – 0 – 10	0 – 6.2	121 – 180	180 – 146 – 163
20 days; single feeding, 8 A.M. Prot. Ins. 14 and Ins. 8 in A.M.	2.6 – 14.9	124 – 175	
20 days; feedings 8 A.M. & 5 P.M. Prot. Ins. 16 and Ins. 6 in A.M.	0 – 1.9	124 – 165	124 – 160 – 56

*Diurnal blood sugars determined at fasting, one hour after 8:00 A.M. meal, and nine hours after A.M. meal (or before supper).

From thirty-second to fiftieth day, protamine insulin reduced to 10 units. Urine sugar-free. Diurnal blood sugar readings*, 137, 120, 29 mgs.; daily slight muscular weakness at 4:00 P.M. Fifty-first day, severe convulsion, relieved by 10 gm. of glucose intravenously.

From fifty-second to seventy-ninth day, protamine insulin continued at 10 units, but diet increased from carbohydrates 100, proteins 100, fats, 90, to carbohydrates 110, proteins 141, fats 113. Urine sugar varied from 1.7 to 9.1 gm. in twenty-four hours. Occasional late afternoon weakness and eye symptoms. Weight increased from 11.3 kg. to 12.7 kg.

Summary.—This animal was placed on protamine insulin alone forty-eight hours following operation. A stormy postoperative course failed to require more than 12 units of protamine. After recovery from infection, 12 units of protamine daily caused at first mild symptoms of hypoglycemia, then convulsions eight hours after injection and feeding, and finally severe convulsions. On 10 units of protamine daily, afternoon weakness was noted, but with an increase in diet, convulsions did not occur, nor was much sugar spilled. Weight and general condition of the animal improved. Once to twice weekly it was noted that the animal had difficulty during the late afternoon in rising to his feet and apparently did not see well. These signs disappeared after food was consumed.

DOG 4.—(Crossbreed N.) Depancreatized Oct. 15, 1936. Protamine insulin, 10 units, was started on the second postoperative day, before a full meal. The urine sugar has ranged from 12 to 14 gm. per twenty-four hours. Protamine insulin, 14 units, has not decreased the glycosuria. General condition is excellent one month after operation. Weight (13 kg.) unchanged.

DOG 5.—(Spaniel A.) Depancreatized May 1, 1936, and placed on insulin 6-0-6. Urine sugar excretion ranged from 0 to 10 gm. in twenty-four hours; fasting blood sugar from 132 to 148 mg. per 100 c.c. Diurnal blood sugars,* 148, 178, 206.

This animal was frequently stuporous but had no convulsions. At 4:00 P.M. the blood sugar was often low. Vomiting occurred several times without apparent cause.

Protamine insulin alone, once or twice daily, with one or two feedings and protamine insulin with accessory insulin, failed to produce stabilization. Fasting blood sugar levels varied from 39 to 201 mg. Semicoma was frequent.

Summary.—This is an example, rare in our experience, of a diabetic dog that cannot be well managed with insulin and careful attention to diet. Protamine insulin did not improve the erratic tolerance.

DISCUSSION

It is generally conceded that dogs with the entire pancreas removed are not easily maintained consistently sugar-free for long periods of time. Our results with two dogs (Nos. 1 and 2) would indicate that it may be easier to maintain this condition with protamine. Dog 2 has been kept sugar-free for three months with 16 units of protamine insulin and 6 units of insulin, both given at 8:00 A.M. with the morning meal. A personal communication to Dr. Ivy from Dr. C. H. Best stated that he has "kept one completely depancreatized dog in excellent condition for nine months on one dose of protamine insulin per day."

While our results in experimental diabetes are in general accord with the clinical reports of other observers, we think that several interesting facts have been brought out in the study of dogs on protamine insulin. This is notably true in Dogs 3 and 4 which have required only 10 to 14 units of protamine insulin in one dose per day since the second day after complete pancreatectomy. Glycosuria has remained at a low level. This is in contrast to the usual dog after operation, in which 20 to 40 units of regular insulin per day have to be given in two doses in order to control glycosuria. Dog 3 unexpectedly had frequent hypoglycemic convulsions and lost weight after operation until the diet was increased by one-third. Although this animal also had a rather stormy postoperative course with infection, 12 units of protamine insulin kept him nearly sugar-free. Recovery was followed by numerous convulsions until the protamine insulin was reduced to 10 units and the diet increased. The ease of control in these dogs by small amounts of protamine insulin supports the clinical observation by Joslin[4] that diabetic patients just starting treatment are apparently more quickly controlled by protamine insulin than by regular insulin.

Experience with Dog 3 also supports the clinical observation that protamine insulin permits a relatively high carbohydrate allowance without an increase in total-insulin units, and particularly that an increase in carbohydrate may actually be necessary in order to avoid hypoglycemic reactions.

Dog 5 presented the problem of labile, fluctuating blood sugar levels with frequent hypoglycemic reactions after small amounts of insulin. She was alternately lively and comatose. The substitution of protamine insulin and a subsequent return to regular insulin caused no improvement. The explanation of the inability to control this dog satisfactorily is not clear. The condition resembles in many respects certain clinical types of unstable diabetes illustrated by Case 6.

The injections of protamine insulin, including preparations containing traces of calcium or zinc, have all been deliberately given in the same site. No harmful effects have been detected.

SUMMARY AND CONCLUSIONS

In six patients with severe diabetes, never adequately controlled with regular insulin, the use of protamine insulin has made more effective management possible. Decisive results have been obtained in three patients who had had persistently high fasting blood sugar levels and morning glycosuria; aglycosuria has

been maintained almost continuously for six to eight months. The total-insulin units have been progressively decreased and the diet increased. Three unstable diabetics have been less strikingly benefited. In two milder cases of this type, satisfactory control has been effected but slight, intermittent glycosuria persists. One very unstable diabetic remains unstable, but on protamine insulin and five feedings daily, reactions are avoided and there is subjective improvement.

Four of five depancreatized dogs were satisfactorily maintained by the use of protamine insulin; two that had previously received regular insulin were more easily managed by the addition of the new preparation. Two dogs placed directly on protamine insulin alone soon after operation required fewer insulin-units than are ordinarily necessary after depancreatization; infection, post-operatively, in one dog failed to increase the protamine insulin requirement more than a slight degree. In one dog with extremely labile blood sugar levels and frequent reactions, stabilization was not achieved with insulin or protamine insulin. No harmful local effects have been noted from injections of protamine insulin made daily at the same site.

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MENINGOCOCCUS ANTITOXIN*

Laboratory and Therapeutic Status

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THE meningococcus (*Diplococcus intracellularis meningitidis*) is an organism of the invasive type. In its usual rôle, as an etiologic factor in cerebrospinal meningitis in the human being, its portal of entry, route of infection and approach to the cerebrospinal canal are not definitely known. It is recognized, however, that dissemination of this organism, in many cases, is by the blood stream, and some authorities even go so far as to state that the disease is primarily a blood stream infection and that involvement of the meninges is the result of secondary invasion.

The question before us at this time, while it has a bearing on the primary infection with the meningococcus and its route of invasion, is concerned more especially with the manner in which it produces the symptoms of meningitis and how it causes death and, what is more important, the most scientific and satisfactory method of controlling the infection.

The idea that the meningococcus might produce a specific toxin is not new. The symptomatology of the disease would suggest a toxemia and for many years investigators have been endeavoring, by one method or another, to obtain such a toxin from this organism. And, because extracts and autolysates prepared from it proved to be toxic or poisonous to certain laboratory animals, it was thought that the specific toxin must be a product of cell destruction or disintegration. In other words, it has been generally conceded that if a toxin is involved as an etiologic factor in meningococcus meningitis, it must be an endotoxin. There is no doubt that the meningococcus, when allowed to autolyze in vitro, yields a powerful toxic substance, and that this is more or less specific to the meningococcus. There has never been any positive and decisive evidence to the effect that this is the substance which is responsible for the characteristic symptoms of the disease, as recognized in the human being, nor that it is produced in vivo during infection. Many articles may be found in the literature, as far back as 1906 and 1907, bearing on the question of the relationship of this endotoxin or autolysate to the symptomatology of the disease and to the preparation of therapeutic antiserums from it, but proof is lacking that an endotoxin can stimulate in an animal the production of either a typical antiendotoxin or an antiexotoxin with a potency of any appreciable titer. Therefore, as recovery from meningococcus meningitis almost invariably results in permanent immunity, it is hard to conceive how an endotoxin can be responsible for

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the immune bodies involved, when it cannot be definitely shown experimentally that this same endotoxin is capable of stimulating these immune bodies. A toxin more specific and more in the nature of an exotoxin would be required for the production of this type of immunity.

In the preparation of antimeningococcus serums for therapeutic purposes animals have variously been injected subcutaneously, intramuscularly and intravenously with suspensions of live or dead bacteria, with autolysates or extracts and recent evidence seems to confirm the generally accepted idea that therapeutic results are obtained by them by virtue of their antibacterial properties and not as a result of neutralization of any so-called endotoxin.

As early as 1925, while working with broth cultures of the meningococcus, I found that comparatively young cultures of the meningococcus, in fairly large flasks of suitable liquid media, would almost invariably develop with pellicle formation.[1] It was discovered at that time that the filtrates from these luxuriant young cultures contained a toxic substance, specific for the four individual Gordon types respectively, differing entirely from the toxic substance prepared as an endotoxin and possessing properties characteristic of a true soluble toxin.[1, 2] The most outstanding property of this new toxin appeared to be its ability to stimulate the formation of a true antitoxin also specific against the four types, respectively. It was found that while this toxin was not very poisonous for laboratory animals it was extremely toxic for the human being, especially upon subcutaneous or intracutaneous injection.[1, 6] It was also discovered, as the cultures were allowed to remain longer in the incubator and as more of the organisms were autolyzed, that the filtrates had become more poisonous for animals and apparently just as toxic but less specific for the human. In other words, in young cultures, the exotoxin predominates over the endotoxin while in older cultures the reverse is true. The reason for this becomes apparent when it is realized that the endotoxin is a product of disintegration of the older bacterial cells while the soluble toxin is the result of the vital activity of the young growing organisms. Although the filtrates of young cultures, taken at a time when autolysis was negligible, were but slightly toxic for laboratory animals, except when injected intracisternally,[5] it was found that when diluted many hundred times, and injected intracutaneously, they would produce positive skin reactions in a certain percentage of human beings.[1, 6] This was to be expected because the human being, as far as we know, is the natural host of the meningococcus and, as such, is the most susceptible animal to this organism as well as to its specific soluble toxin. Autolysates and endotoxins prepared mechanically when injected at the same time gave negative results.[1]

Further experiments were reported to the effect that active immunity to the toxin can be stimulated in the human being following injection of the soluble toxin.[6, 13] Actual tests showed that individuals susceptible to the skin test dose of toxin can be made resistant to this same dose of toxin by repeated subcutaneous injections of the undiluted toxin and suggest a possible means of active protection against the disease. It was also found, when a filtrate containing the specific toxin for any individual type, was mixed with a diluted homologous antitoxin or with a diluted homologous meningococcic meningitis convalescent serum, that it would produce negative skin reactions in individ-

uals known to be susceptible to the toxin in question.[1] These phenomena of neutralization of the filtrates or toxins with both the antitoxin and convalescent sera were considered very significant of the specificity of the toxin as well as of the antitoxin.

Carrying this work still further, it was found that this antitoxin would not only prevent the specific toxin from reacting in the human being, but would protect a large percentage of monkeys after they had been inoculated with known fatal doses of the live virulent meningococcus.[3, 4] In carrying out these experiments, the animals were first inoculated intrathecally with lethal doses of the live organism and later, when symptoms indicative of a fatal issue appeared, were injected intraperitoneally with the antitoxin. Eighty per cent of the treated animals recovered while 100 per cent of the controls died. These experiments were controlled with monkeys inoculated as above with cultures alone and also with monkeys treated in the same way with an antibacterial serum.

Another interesting finding in connection with our experiments on monkeys, which also points very strongly to the specificity of the soluble toxin and its ability to stimulate active immunity both in the monkey and in the human being, was the fact that intracisternal injections of this toxin produced symptoms typical of meningitis in the monkey, with death in some animals, while those that recovered were found to be immune to the lethal dose of the live organism.[4]

The results of the experiments on monkeys which showed that an antiserum with high antitoxic content, when introduced into the body far removed from the focus of infection, could bring about recovery in so large a percentage of cases,[3] while an antibacterial serum given in the same manner failed to protect,[4] coupled with the fact that injection of the soluble toxin alone would produce typical symptoms of the disease,[4] naturally led to the conclusion that recovery was due to neutralization of the specific exotoxin and not to a direct bacteriolytic effect on the organisms themselves. This is especially true, since blood cultures and cultures from the spinal fluid of some of these monkeys were found to be positive after it was apparent from a lack of symptoms that the animals had entirely recovered.[3] These results, together with others on laboratory animals which showed that symptoms of meningitis could be produced with the soluble toxin alone, were taken as a measure of proof that the symptoms of this disease and death are due to the specific exotoxin secreted or excreted into the surrounding tissues, and not necessarily to an endotoxin liberated only upon the death and disintegration of the bacteria.

Applying this method of treatment, as carried out in the monkey, to the disease as found in the human being, the results of Hoyne[7, 8] and others[11, 12] have confirmed those following the experiments on the monkey, and have shown that the neutralizing effect of the antitoxin on the specific toxin is more essential and more certain than the bacteriolytic effect of the antibacterial serum on the organisms themselves. Intravenous injections of the antitoxin with and without intraspinal or intracisternal injections in a large number of cases, some of which have been reported[9-11] and others to be reported in the near future,[12] controlled at the same time with a similar number of cases treated in the usual manner with an antibacterial serum, have lowered the previous mortality rate

on an average of from 25 to 50 per cent. It has been invariably reported that hospitalization has been shortened by several days and complications have been greatly reduced. It is also very apparent that patients seem to be more comfortable immediately following injection with the antitoxin, doubtless due to neutralization of the circulating toxin with the antitoxin. In one series of early cases[8, 9] and others,[11] some of which have not as yet been reported,[12] where intravenous injections were given alone without resorting to spinal puncture for diagnosis or for relief of pressure, diagnosis being confirmed by blood culture, there was a mortality rate of 25 per cent. The previous mortality rate and the rate of control cases treated at the same time with antimeningococcic serum prepared as an antibacterial serum was 45 per cent. In another section of the country where an epidemic was in progress with a mortality rate as high as 70 per cent among patients improperly treated or not treated at all, it has been very forcibly shown,[12] when diagnosis was made early and when proper treatment with meningococcus antitoxin was instituted at once, that the mortality rate could be greatly reduced. In one locality where such methods were carried out, many cases were practically aborted and the mortality rate was reduced to less than 10 per cent.* In the cases cited above, as well as in all cases where the antitoxin was used with favorable results, stress has been laid on the desirability of large doses given early and injected intravenously, irrespective of what other route of injection was being used at the same time or what other treatment was being employed.

DISCUSSION

Evidence has been submitted to the effect: that the meningococcus produces a soluble toxin, specific to the various types of the organism, which stimulates the production of homologous antitoxin in susceptible animals; that neutralization of the toxin takes place in vivo as well as in vitro; that recovery from meningococcic meningitis follows intravenous injections of the antitoxin and, irrespective of the invasiveness of the organism; that antitoxin therapy is logical, scientific and effective.

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THE EFFECT OF ATROPINE ON THE GASTRIC CONTENTS OF MAN*

Statistical Report of One Hundred Seventy Cases

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OUR knowledge of the action of atropine on the stomach is based chiefly on the results of experiments on animals. There is evidence that atropine is capable of arresting both the psychic[1] and intestinal[2] phases of gastric secretion. With respect to obviating the former in human experiments, some observers have introduced a liquid test meal through a duodenal tube literally behind the patient's back. Atropine is also *partly* able to control the chemical phase of gastric secretion.[3] On the secretion induced by secretin and histamine, atropine is capable of acting only if the amounts of the former are definitely limited in quantity. Even a toxic dose of atropine cannot arrest the effect of a large dose of gastrin or histamine.[4]

Bylina[5] showed also, contrary to the failure of earlier observers,[6, 7] that atropine can control to some extent the pancreatic secretion caused by introducing HCl into the duodenum. With respect to the action of atropine on a test meal, pouch experiments[4, 8, 9] show an unequivocal action of atropine; but the dose must be optimal and must be administered at an optimal time,[9] presumably because as the hormonal phase of secretion becomes well developed atropine becomes less capable of arresting secretion. If we add that in a general way, gastric secretion is not much altered when the stomach is severed from the nervous system,[10] and that atropine acts on a subcutaneous pouch which consists of transplanted mucosa and submucosa[9] in which only Meissner's plexus is intact, we have the main basis for the statement that the seat of action of atropine is peripheral; it is believed to act on the vagal neurocellular junction principally, whereas the secretin bodies are believed to act principally on the gland cell itself.[8, 11]

The results of the action of atropine as judged by gastric analysis, in which conditions are different from a pouch experiment, are confusing. The principal difficulties are the following: (1) atropine may affect the emptying time of the stomach, although observers are about equally divided that it hastens or retards

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gastric emptying time;[12] (2) there may be a possible effect on the biliary system and on duodenal regurgitation;[13] a possible effect on duodenal secretion through an effect on the vagus when the blood sugar level is altered;[14] (4) a possible stimulating action of atropine;[15] (5) it must be remembered that gastric secretion and the gastric contents are by no means synonymous; however, the former may apparently be calculated from the latter;[16] (6) all the difficulties met with in animals, including dosage, time of administration, and nature of the stimulus, must be taken into account.

The number of observers, who, in gastric analysis, obtained no effect, a varying effect, or an increase of gastric secretion, one should more properly say gastric contents, after atropine[17] is about equal to those who obtained a decrease after atropine.[18] Some therapists have felt that atropine was effective only when administered in toxic doses, or have denied its value in controlling gastric secretion.[19]

There appeared to be no studies in the literature which had been subjected to statistical analysis. For this reason it was thought that if a sufficient number of patients were treated in this manner the augmenting power of atropine on the gastric contents could be evaluated. Grain $\frac{1}{75}$ of atropine hypodermically was purposely chosen as a very moderate dose. Two simple experiments were carried out on 170 patients.

Experiments.—(1) (a) In 170 patients after a six-hour fast, the fasting contents were removed, measured, and titrated. (b) In the same patients the same procedure was carried out, except that $\frac{1}{75}$ gr. of atropine sulphate was administered hypodermically one-half hour before the removal of the fasting contents. (2) (a) After the fasting contents had been removed, 105 patients were given a control test meal of 350 c.c. of water and 6 crackers. The meal was removed in one hour. (b) In the same patients the same procedure was carried out, except $\frac{1}{75}$ gr. of atropine hypodermically was administered one-half hour before the meal was started. It should be noted that the meal was also removed in one hour, but that the atropine had been acting for one and one-half hours at the time the meal was removed.

Statistical Considerations.—Before detailing the results, the statistical methods used in this study may be outlined.[20] Since the nature of the gastric secretion is partly a function of sex and age, it may be objected that in this study these were not considered. Since, however, the same patients were used for control and experiment, the comparison of "before" and "after" atropine is perfectly legitimate. As a matter of fact the 170 cases were divided into 10 groups of 17 each, and it was found that the averages of the groups did not differ significantly from the general average (intraclass correlation method of Fisher). In a study of this kind the significance of the statistical results may be judged as follows: (1) The average difference before and after atropine must be compared to the irregularity of the secretion, i.e., the mean difference is divided by the standard error of the difference. This gives Fisher's "t." For the number of experiments here performed a value of $t = 2.0$ may be considered borderline, it signifies that only 5 times out of a hundred would the same result be secured by mere chance, and may be written $p = 0.05$. For a value of $t =$ less than 2, which has no significance, $p > .05$; for a value of $t = 2.6$

$p = .01$; i.e. by coincidence the same result would occur only once in a hundred times. Such a result may be regarded as significant. For t greater than 2.6, $p < .01$, such a result is highly significant. (2) The above given values take it for granted that the "curve" from which the result has been calculated is normal. In the present study the curves were also analyzed. Fisher's g_1 analyzes the curve in a lateral direction, for asymmetry called skewness, and g_2 in a vertical direction, virtually equivalent to kurtosis. Abnormality of the curve is indicated when g_1 and g_2 are 2.5 times or more greater than their respective standard errors. When this is the case, conclusions drawn from the value of t must be accepted with caution. (3) It is possible to analyze a curve accurately by calculating the formula of the curve.

Something should be said about the number of increases and decreases after atropine. Thus out of 170 cases the volume of the fasting contents was decreased 120 times and increased 50 times. This fairly large number of increases has been used as an argument for the augmenting power of atropine. The actual number of changes must be looked at in the light of the irregularity of the change. This is done by dividing the average change by the standard deviation of changes, known as x over sigma, and seeking out this value in a table. In the present instance the Table I shows 54 increases to be expected. Only 50 were found; the difference is probably due to the irregularity of the curve as shown by g_2. If the curve had been regular, however, then the difference could have been ascribed to the experimental conditions.

TABLE I

	FASTING CONTENTS			TEST MEAL		
TOTAL CASES TESTED	170	167	167	105	104	104
	VOL. C.C.	FREE ACID	TOTAL ACID	VOL. C.C.	FREE ACID	TOTAL ACID
Before atropine Vol. c.c.	59.16			107		
Free acid (degrees)		28.0			23.9	
Total acid (degrees)			47.7			48.47
After atropine						
Average change†	−17.39	−2.85	−4.1	+41.5	−3.15	−3.51
t	4.4*	1.5	2.1	3.96	2.2	1.6
p	<0.01	>0.05	<0.05	<0.01	<0.05	>0.05
No Change In Zero Acidity (No. Cases)		30			9	
g 1.	0.0353*	0.3568		−0.175	0.6853	
St. error g 1.	0.188	0.21		0.24	0.234	
g 2.	2.52*	0.3874	1.58	−0.21		
St. error g 2.	0.38	0.41	0.41	0.48		
Expected No. increases	54	55	70		41	45
Number found	50	55	68		42	40
Expected No. decreases				37		
Number found				43		

*Indicates the results from a geometric curve.
†For an explanation of p, t and g see text.

Results.—(1) *Fasting Contents:* (a) *Volume:* The volume of the fasting contents was reduced 17.4 c.c., from 59.2 c.c. to 41.8 c.c. The curve which yielded this result was abnormal, g_2 exceeded its standard error by about 9

times, to give a "t" of 4.8. A somewhat better curve, obtained by geometric proportions, in which g_2 exceeded its standard error by 6½ times, reduced t to 4.4. Under these circumstances it seems unlikely that t would have been reduced to insignificance in a normal curve, and the present result may with practical certainty be considered highly significant. (b) *Acidity:* In considering the free acidity, it is desirable for mathematical reasons to note especially the "zero" acidity. Out of 167 available cases, it was found that before atropine there were 46 cases of zero acidity, after atropine 40 such cases. This is not a significant decrease. There were 30 patients with zero acidity before and after atropine. If these are excluded, the remaining 137 cases may be looked upon as having produced the free acidity both before and after atropine. The free acidity was 28 degrees before, and 25 degrees after atropine. This decrease was found to be not significant. (c) *Total acidity:* This was reduced from 48 degrees before, to 44 degrees after atropine. T was 2.1, about on the border line of significance, but the curve was abnormal; a somewhat better but not normal geometric curve increased t to 2.2. It may be concluded that no significant decrease of total acidity was demonstrated.

(2) *Test Meal:* (a) *Volume:* The volume of the meal was increased 41.5 c.c. from 107 to 148.5 c.c. This increase was found to be highly significant. (b) *Free Acidity:* This was found to be decreased 3 degrees from 24 degrees before atropine. Following the methods outlined above, this result was considered not significant in an abnormal curve. (c) *Total Acidity:* This was decreased 3.5 degrees from 48.5 degrees before atropine. This result was considered not significant.

SUMMARY AND CONCLUSIONS

It is pointed out that in dogs with pouches, atropine is capable, if properly exhibited, of decreasing the volume, and also the acidity of a test meal, and more easily of the psychic and intestinal phases of gastric secretion. In human beings the gastric contents, especially after a meal, have been found often to be increased in volume, with the acidity changes variable. One hundred seventy patients were examined for change in fasting contents and 105 for change in test meal, after 1/75 gr. of atropine had been allowed to act, respectively one-half and one and one-half hours. The volume of the fasting contents was reduced 17.4 c.c., the volume of the test meal was increased 41.5 c.c. These values were found to be statistically significant and checked in curves which were analyzed. The acidity was not materially affected in these experiments, except so far as changes in volume concentrated or diluted the acid. The possible causes for the increase of gastric contents when atropine is given to a patient digesting a meal are briefly outlined. These causes have not yet received adequate investigation in relation to the present problem.

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OBSERVATIONS ON INDUCED ENCYSTMENT OF ENDAMEBA HISTOLYTICA IN VITRO*

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PREVIOUSLY, it was reported that when either trophozoites or washed cysts of *Endameba histolytica* were introduced into the S.C. medium,[1] various stages of development of the parasites were observed during the course of the incubation period, i.e., excystation, metacystic development and encystation as well as division. The extent of encystation as seen in the culture medium, however, has been more or less variable and at times quite inconsistent. In no instances were encysted forms found to predominate over the other developmental stages of the parasites that were present simultaneously in the culture medium. The addition of an excess of rice starch to the medium prior to an exposure to room temperature as suggested by Dobell[2] did not seem materially to increase the percentage of the encysted forms in the culture medium. Cleveland and Sanders,[3] however, reported that encystment could be successfully induced by the use of Loeffler's serum medium.

This paper is to describe observations on induced encystment of *Endameba histolytica* by the use of the S.C. medium as the primary culture and secondarily to determine the viability of the cysts thus produced in vitro as well as in vivo experiments.

MATERIALS AND METHODS

The cysts of *Endameba histolytica* used in this study were obtained from a carrier detected among Freshman medical students. Washed cysts were prepared according to the method of Hegner[4] and were found to contain considerably fewer bacteria than were present in the original stool.

Method of Inducing Encystment.—The medium used in inducing encystment of *Endameba histolytica* is Loeffler's slant covered with Ringer's solution.

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A 4 mm. loopful of rice starch sterilized by dry heat at 180° C. for forty-five minutes is added to the medium. Encystment is brought about by transfering a portion of sediment containing many trophozoites in the S.C. medium to the encystment medium. The tube is then incubated at 37° C.

Preparation of Washed Cysts from Encystment Medium.—The supernatant fluid from several tubes of Loeffler's slant is decanted and the sediment containing the cysts is collected. After an addition of sufficient amount of sterile normal saline, the sediment is washed by means of centrifuging at 1500 r. per minute for several minutes. This process is repeated at least three times in order to eliminate as many bacteria as possible. The washed cysts are suspended in Ringer's solution and stored in an ice box until used.

Determination of Viability of Induced Cysts.—The washed cysts prepared by the above method were left at the room and refrigerator temperatures respectively and their viability was determined at various time intervals by subculturing a portion of the sediment to the S.C. medium. In determining the viability of cysts in vivo, young kittens and albino rats (about eight weeks old) were used. These animals were fed the cysts on empty stomach (2 and 4 c.c., respectively, each cubic centimeter containing approximately 250,000 cysts) and the animals placed under observation*

OBSERVATIONS

By the use of the method above described, the maximum encystation was found to occur, as a rule, after an incubation period of from forty-eight to fifty-four hours at 37° C. Encystment was apparently absent during the first twenty-four to twenty-eight hours of incubation during which time trophozoites only were present. Cysts, however, began to appear when about thirty hours' incubation period was reached. From this time on a gradual increase in the number of encysted forms had been noted. On further incubation, however, the cysts became noticeably fewer in numbers and at ninety-six hours' incubation cysts were seldom found. The induction of encystment by this means has been found to be quite consistent. The number of the cysts predominated to the extent of from 70 to 85 per cent of the total organisms present in the culture medium. The cysts, furthermore, were found to be normal in every respect and would excyst normally when introduced into the S.C. medium as well as other suitable mediums, particularly that of Tanabe and Chiba.[6] The viability of cysts persisted for as long as forty to forty-five days at refrigerator temperature and for six to eight days at room temperature. On the other hand, washed cysts prepared from the original stool of the carrier were found to maintain viability for fifty-five to fifty-seven days at 5° C. and for eight to nine days at 22° C.

Attempts were made to determine whether the repetition of the induction of encystment would influence the extent of encystment in the medium. The actual experiment showed that it resulted in a gradual reduction in the number

*Recently, I have reported that the infectivity of the washed cysts of *Endameba histolytica* for albino rats apparently varied with the feeding time of the animals. Thus, when the animals were fed with the cysts on empty stomach, the rate of infectivity was found to be higher than when the stomachs were full.[5]

of cysts as inductions continued, and after several such transfers a total or partial disappearance of cysts ensued. It is quite probable that successive transfers of the organisms may lead them to acquire an apparent degree of relative tolerance to the cultural conditions to which they have been subjected.

Relative proportions of cysts at various stages of the development were studied at two day intervals after the cysts were exposed to refrigerator temperature. Three hundred cysts taken at random were studied each day. These cysts were stained by Heidenhain's iron-hematoxylin method and by the method recently described by me.[7]

TABLE I

RELATIVE PROPORTION OF 1, 2, AND 4 NUCLEATED CYSTS DETERMINED AT TWO DAY INTERVALS AT THE REFRIGERATOR TEMPERATURE

NUMBER OF DAYS	NUMBER OF CYSTS EXAMINED	UNINUCLEATES		BINUCLEATES		QUADRINUCLEATES	
		NUMBER	%	NUMBER	%	NUMBER	%
Control*	300	186	62	60	20	54	18
2	300	180	60	57	19	63	21
4	300	156	52	69	23	75	25
6	300	75	25	96	32	129	43
8	300	15	5	117	39	168	56
Total	1500	612	40.8	399	26.6	489	32.6

*Forty-eight hours' incubation at 37°C.

As will be seen from Table I, there was a gradual diminution of uni- and binucleated cysts and a simultaneous increase in the number of quadrinucleated cysts as the time of exposure was prolonged. Thus, on the eighth day of exposure to refrigerator temperature the relative proportion of uninucleated cysts to quadrinucleated became 5:56 whereas on the first day it was 62:18.

TABLE II

RELATIVE PROPORTION OF CHROMATOID BODIES IN 1, 2 AND 4 NUCLEATED CYSTS DETERMINED AT TWO DAY INTERVALS AT THE REFRIGERATOR TEMPERATURE

NUMBER OF DAYS	NUMBER OF CYSTS EXAMINED	CYSTS WITH CHROMATOID BODIES						
		TOTAL	UNINUCLEATES		BINUCLEATES		QUADRINUCLEATES	
			NUMBER	PER CENT	NUMBER	PER CENT	NUMBER	PER CENT
Control*	300	153	75	49.0+	48	31.3+	30	19.6+
2	300	138	66	47.8+	51	36.9+	21	15.2+
4	300	117	53	45.2+	31	26.5+	33	28.2+
6	300	51	16	31.3+	15	29.4+	20	39.2+
8	300	9	6	66.6+	3	33.3+	0	0
Total	1,500	468	216	46.1+	148	31.6+	104	22.2+

*Forty-eight hours' incubation at 37°C.

As shown in Table II the presence of chromatoid bodies within the cysts likewise showed a decided reduction by the eighth day, this being 3 per cent as contrasted with 51 per cent in the control tube. Craig[8] recognized the occurrence of chromatoid bodies in at least 50 per cent of all cysts of *Endameba histolytica* and claimed that they were most numerous in immature cysts. Yorke and Adams,[9] on the other hand, found that they were most frequently found in bi- and quadrinucleated and rarely in uninucleated cysts. In this study, of the total of 468 induced cysts containing chromatoid bodies, 216 were uninucleated, 148 binucleated, and 104 quadrinucleated.

An exposure of the induced cysts to room temperature was marked by a gradual development of immature cysts to maturity within twenty-four hours, but as several days elapsed, only a small and rapidly decreasing proportion of the cysts was found to be living. An exposure to incubator temperature (37° C.) brought about an apparent degeneration of cysts, and within twelve to twenty-four hours the majority of them either underwent disintegration or became "necrobiosed" as aptly termed by Garcia.[10] Previously, Hegner, Johnson and Stabler[11] as well as myself[12] independently found that the development of immature cysts to maturity occurred at room and refrigerator temperatures, but not at incubator temperature.

When the washed induced cysts consisting predominantly of uninucleates were introduced into the S.C. medium, a good growth of the organisms was obtained at the end of twenty-four hours' incubation. This seems to indicate that immature cysts as well as matured ones were capable of excysting in a suitable medium, provided conditions of temperature and moisture were favorable for the development of the organisms.

Not infrequently in the S.C. medium, particularly when starch granules were too few to be detected in the microscopical fields, the amebas presented a more or less shadowy appearance with an apparent loss of motility, this may be appropriately called "hungry ameba." These amebas may be made actively motile by an addition of rice starch to the culture medium, followed by incubation for half an hour at 37° C. Such amebas, however, as a rule, were found to be unsuitable material for inducing cyst formation. Incidentally, the presence of "hungry ameba" may become a source of error in the cultural diagnosis of amebiasis. A seemingly negative finding may, therefore, be converted into a positive one simply by the addition of rice starch to the culture medium.

The results of animal experiments revealed that 2 out of 3 kittens fed by the stomach tube with washed induced cysts became infected on the sixth and seventh days. Daily examinations of stools of these infected animals showed many active trophozoites that were cultivable in the culture medium, but no cysts were found at any time during the course of the experiment. One of the animals developed pneumonia and died on the thirty-sixth day. At autopsy, many trophozoites were recovered from the large intestine, but no pathologic lesions were recognized. This was a decided contrast to the conditions observed in the control animal infected with washed cysts of the original stool of the carrier. The animal died on the eighteenth day with the characteristic lesions throughout the large intestine.

The consistency of stools of the two infected kittens varied from day to day, but usually the stools were semiformed with little or no mucus or blood. The trophozoites recovered from the colon were capable of encystment in vitro, although they did not encyst in the intestines of the kittens.

Of 10 albino rats used for experimental amebiasis, only two became infected and the 8 remaining animals were apparently ameba-free as found by repeated examinations of postcathartic stools. The infected animals were killed on the forty-sixth day and no lesions were found in the large intestines. Furthermore,

a spontaneous disappearance of cysts occurred on the twenty-seventh and thirty-sixth day of the infection. This finding, in general, confirms the previous results.[5]

COMMENT

The probable mechanism of encystment of *Endameba histolytica* in vitro as well as in vivo has not as yet been explained. Among the hypotheses advanced, a radical change of environmental condition (either chemical or physical) as controlling factors in encystation may be cited as the most plausible one. Dobell and Laidlaw[13] observed that the organisms were often found to become encysted upon removal from a starch-free medium to one containing this substance. As early as 1918, Cutler[14] claimed that encystation of the organisms was induced by placing the culture in a refrigerator for a few days or without subculturing for four to five days. His observations, however, were not supported by Boeck and Drbohlav[15] as well as Dobell and Laidlaw.

The constituents of the primary and encystment mediums used in this study are so different that a radical change in cultural conditions may be initiated. Upon transfer of the sediments from the primary to encystment mediums, the bacterial flora may be subjected to variations. It is quite probable that certain species may eventually replace or crowd out those originally transferred from the primary culture medium. This may render environmental conditions unfavorable for the existence of trophozoites; hence encystment would be a natural outcome of the situation.

The failure of washed induced cysts to bring about the pathologic lesions in the kittens despite the presence of the parasites in their intestines may partly be explained on the basis of individual differences in resistance, a state perhaps analogous to that observed in human carrier. Or else the constituents of the mediums may partly be responsible for the variations. According to Dobell and Laidlaw, a strain of ameba known to be pathogenic to a kitten may be made nonpathogenic by culturing the organisms in starch-containing media. It has been also my observation in the past that several strains of amebas definitely pathogenic to kittens frequently lost their infectivity after having been cultured for several generations in the S.C. medium. That the bacterial flora accompanying the amebas in the original stool may play an important role in determining the production of the pathologic lesions in the animals was exemplified in the experiments of Spector.[16] She demonstrated that the degree of pathogenicity of *Endameba histolytica* to kittens apparently varied with the type of the bacterial flora found in the intestines and concluded that the various species of bacteria produced marked variations in the virulence of different strains of the amebas and in the amount of pathology produced after the inoculation of such strain and bacteria.

SUMMARY

1. The encystment of *Endameba histolytica* in vitro was induced by the growth on the S.C. medium followed by a transfer to Loeffler's medium with Ringer solution and starch. The results of the method have been quite consistent; usually from 70 to 85 per cent of all the organisms have undergone encystation.

2. Observations were conducted to determine the viability of induced cysts in vitro. It was found that the cysts were capable of excysting in suitable culture mediums and that they remained viable for as long as forty to forty-five days at refrigerator temperature and for six to eight days at room temperature. The previous observations that immature cysts may mature in vitro have been verified in this experiment.

3. Induced cysts were found to be incapable of inducing pathologic lesions in kittens or albino rats. In two kittens no symptoms developed despite the presence of the parasites (trophozoites only) in their intestines—a state perhaps analogous to that observed in human carrier. Trophozoites recovered from the large intestines were readily made to encyst upon induction. This seems to indicate that the amebas do not encyst in the intestines of kittens as generally believed, whereas in vitro this is apparently possible. In the two rats which became infected, no characteristic lesions were observed at autopsy. In these animals both trophozoites and cysts were found and the latter spontaneously disappeared during the course of the experiments.

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A MODIFICATION OF THE KRASNOGORSKI METHOD FOR STIMULATING AND MEASURING THE SECRETION FROM THE PAROTID GLANDS IN HUMAN BEINGS*

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DURING the course of studies on conditioned reflex activity in psychoneurotic patients, a procedure has been developed for stimulating, measuring and recording the secretion of the parotid glands. The procedure is based on the Krasnogorski[1] method with certain modifications.

The method is briefly as follows: The secretion of the parotid glands is transmitted to the measuring and recording apparatus by means of two Krasnogorski capsules (Fig. 1) which are held in place over Stenson's ducts by suction. The saliva is collected in a bottle which at the start of each experiment is completely filled with saline (saline reservoir bottle, Fig. 2). The saline, which is thus displaced from the bottle in a volume equivalent to that of the secreted saliva, passes through a tube with a capillary tip, from which it drops into a buret. Each drop of saline closes an electric circuit between two platinum wires sealed horizontally into the walls of the buret below the capillary tip, causing an electromagnet to signal the passage of the drop on the kymograph record. This device for closing the electric circuit is extremely simple in construction and operation, and has the advantage of eliminating the sticking of drops between the electrodes, a difficulty frequently interfering with the accuracy of recording the secretion in the Krasnogorski method. The drops are collected in the buret for the measurement of the total volume of saline displaced during a given experimental period.

In the present studies, acid solutions, most commonly lemon juice, are used as the unconditioned stimulus, in contrast to the solid food used in most of the Krasnogorski studies on children. In working with adult patients it was thought advisable to use a stimulus that would not be dependent upon such cooperation as would be required for the patient to open his mouth to receive solid food. The fluid is injected through two narrow rubber tubes which are held in place in the subject's mouth by being fastened to the capsules (Fig. 1). In this way the stimulus can be administered without requiring the subject to open his mouth. Jaw movements connected with the intake and swallowing of the fruit juice are not recorded.

APPARATUS

Capsule.—Our capsules were reproduced by a local silversmith from a sample kindly given to us in Leningrad by Professor N. I. Krasnogorski, who

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has published a detailed description of the device.* It is a small round silver apparatus, consisting of two concentric silver chambers, an inner circular chamber, 9 mm. in diameter and 3 mm. deep, which is uncovered, and an outer annular chamber 2 mm. wide and 3 mm. deep, which has a silver cover with 20 to 25 perforations. The chambers are completely separated by the partitioning wall and have separate silver outlet tubes which are 7 mm. long and 1.7 mm. in diameter. The outer annular compartment serves as the suction chamber, and is connected with the source of negative pressure through its own opening, marked suction tube (Fig. 1). The perforated cover, through which the suction is transmitted to the mucous membrane around Stenson's duct, was designed to provide an even distribution of the suction. The secretion from the parotid

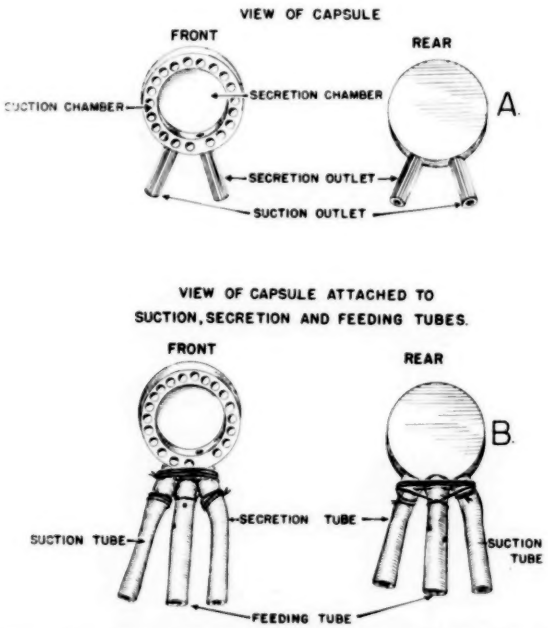

Fig. 1.—The Krasnogorski capsule with suction, secretion, and feeding tubes. The tube for the feeding system is tied to the suction and secretion outlet for support.

gland collects in the inner circular chamber, flowing out to the recording apparatus through the outlet, marked secretion tube (Fig. 1).

Suction System.—The suction is developed by means of a foot pump (Central Scientific Co. 90020) which is connected to a 5-gallon bottle serving as a suction reservoir. The reservoir bottle is in turn connected to a mercury manometer, and through a T-tube, to the suction tubes of the capsules. It is important to have the negative pressure which holds the capsule in place controlled within specified limits. We find that if the suction is less than 24 cm. of Hg the capsule may become dislodged, and if, on the other hand, the pressure exceeds 34 cm. of Hg the mucous membrane may be irritated. These suction readings represent the difference between the current atmospheric pressure and the pressure in the suction reservoir. In our experience a single evacuation of

*This capsule resembles closely the drainage tube devised independently in 1916 by K. S. Lashley[2] for studies on the salivary reflexes.

the bottle to the desired pressure level is sufficient to keep the suction within the proper limits throughout the course of an hour's experiment. This suction apparatus has the advantage of simplicity and economy as compared with that used in the Krasnogorski experiments.

Recording System.—The secretion of the two parotid glands issuing from the secretion tubes of the two capsules is combined by means of a narrow Y-tube, which leads to the short arm of the saline reservoir bottle (*R*) (Fig. 2) containing a 4 per cent solution of sodium chloride. The connections between the capsules and the Y-tube are made of rubber so-called spectacle tubing, 1.5 mm. in diameter. The connection between the Y-tube and the saline reservoir is made with a wider rubber tubing. The distance between the Y-tube and the

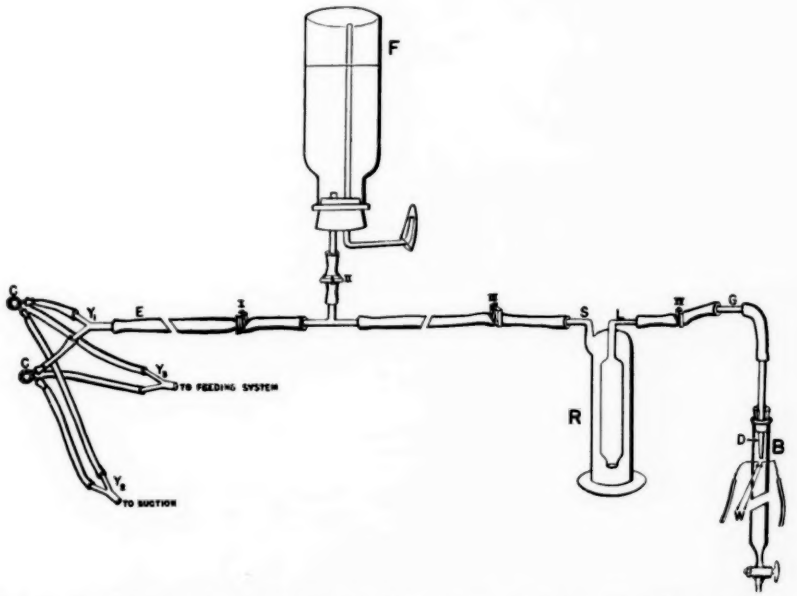

Fig. 2.—Arrangement of apparatus for displacement of saline by saliva. Saliva, collecting in the inner chambers of the capsules (*C*), and filling the Y-tube (Y_1), displaces saline from the saline reservoir bottle (*R*) which it enters via the short tube (*S*). The displaced saline leaves the reservoir bottle through the long tube (*L*), passes through the glass tube (*G*) and drops into the burette (*B*) from the capillary dropper (*D*). Each drop closes an electric circuit between the platinum wires (*W*) sealed into the burette walls. The filling bottle (*F*) and Hoffman clamps *I*, *II*, *III*, and *IV* are used to fill the system with saline before each experiment. The Y-tube (Y_2) connects the capsule to the suction system. The Y-tube (Y_3) connects the feeding tubes which are attached to the capsules to the feeding system.

saline reservoir is considerably longer, about 3 meters, since the patient is in a sound-proof room, whereas the saline reservoir and the recording apparatus are in an adjoining room.

The saline displaced from the reservoir bottle by the secretion, drops into a 50 c.c. buret from a tube (*D*) with a capillary tip (*T*). This tube is supported within the buret by means of a stopper with an air vent. The bore of the capillary tip is selected so as to give drops of an appropriate size, usually about 24 drops per c.c. Small drops have the obvious advantage of giving larger numbers for use in studying the progress of the conditioning experiments. In patients who secrete copiously, a tube with a wider capillary tip must be used

to prevent the drops from merging into a stream. In order to avoid secretion of saliva against a hydrostatic pressure, the level of the capillary tip should be about the same as that of the capsule in the subject's mouth.

In falling, each drop of saline makes contact with two platinum wires (WW) sealed horizontally through the walls of the buret about 2.5 cm. below the capillary tip. The ends of these wire electrodes curve slightly downward and are about 1 mm. apart.

The electrical connections are shown in Fig. 3. The source of E.M.F. is the 110 V. D.C. house circuit, with a 4700 ohm. series resistance. From the resistance the line leads to the platinum electrodes, and then to a wire wound single spool electromagnet with a resistance of 1,000 ohms. A two-microfarad condenser is connected across the platinum electrodes. As long as the electric circuit is incomplete, i.e., when there is no saline drop making contact with the

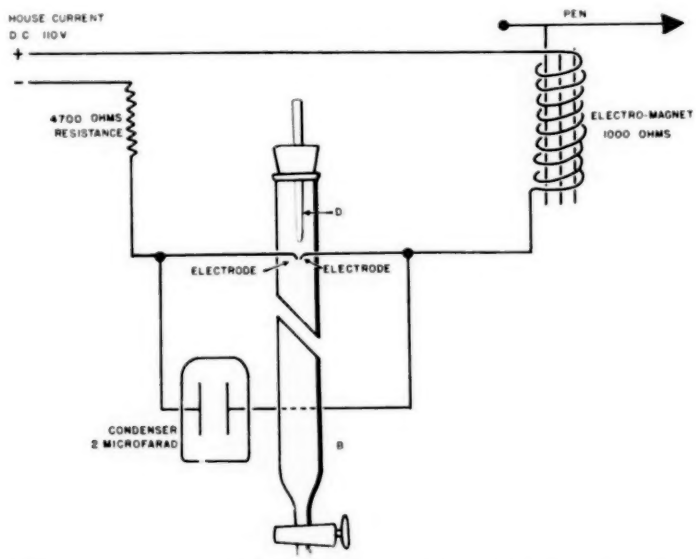

Fig. 3.—Electric connections for measuring and recording the salivary secretion.

electrodes, a pen attached to the magnet traces a straight line on the kymograph record. When a drop of the 4 per cent saline passes between the platinum electrodes the electric circuit is completed and current passes through the magnet. The pen moves away from the base line, returning immediately as the drop passes beyond the electrodes, when the circuit is again broken. Each excursion of the pen thus represents a drop of displaced saline. Coalescence of the drops to a continuous stream is at once evident on the record by a prolonged displacement of the tracing from the base line. Should streaming occur a wider capillary tip must be inserted. A telechron timer records seconds. Other pens attached to the electromagnets are available as needed for signals. The speed of the kymograph can be adjusted between 3.6 and 9.8 cm. per minute. A sample of the type of record obtained is shown in Fig. 6.

Feeding System.—A 5 c.c. Luer syringe is adapted with a three-way metal stopcock. When the stopcock is in suitable position fluid can be drawn into the

syringe from a 50 c.c. Erlenmeyer flask, which contains the fruit juice used to stimulate salivation. When the stopcock is reversed, the connection to the Erlenmeyer flask is closed and the connections to the subject's mouth are opened. The tubing from stopcock to subject is divided by means of a narrow glass Y-tube which rests on the subject's chest, into the two feeding tubes leading directly into the subject's mouth. Each feeding tube is made of spectacle tubing and is tied off at the end by means of a thread which also attaches it to the two silver outlet tubes of the capsule (see Fig. 1). When the stimulus is being administered the fruit juice escapes into the patient's mouth through three or four needle-point perforations in the feeding tube about 2 cm. before the point of attachment to the capsule. The connections leading from the stopcock in the apparatus room to the feeding tubes in the soundproof room are made as short as possible and mostly of glass tubing with short rubber joints. The

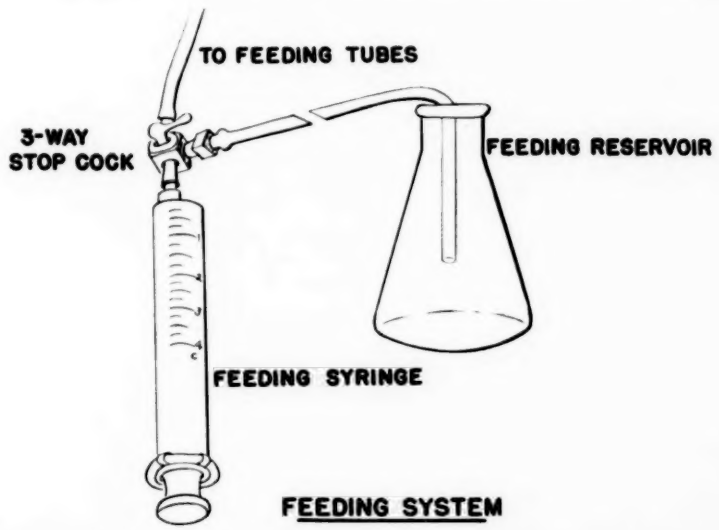

Fig. 4.—Arrangment of apparatus for administering a measured volume of fluid as a stimulus.

whole feeding system from stopcock to subject has a volume of about 25 c.c. This space must be completely filled with fruit juice before the start of each experiment. The needle-point perforations in the feeding tube serve to minimize the possibility of leakage of fruit juice between stimuli. Before each stimulus the amount of juice to be injected, which can be varied according to the requirements of the experiment, is drawn into the syringe from the Erlenmeyer flask, and the stopcock is then turned opening the connection to the subject. As soon as the plunger is pushed in, the fruit juice is sprayed into the subject's mouth through the perforations of the feeding tubes. The time used to empty the syringe determines the duration of the stimulus.

Procedure for Filling Saliva-Saline System.—At the beginning of each experiment the saline system used for recording the flow of saliva (Fig. 2) must be completely filled with a 4 per cent solution of sodium chloride. The saline reservoir bottle (*R*) must be washed free from any saliva remaining from the

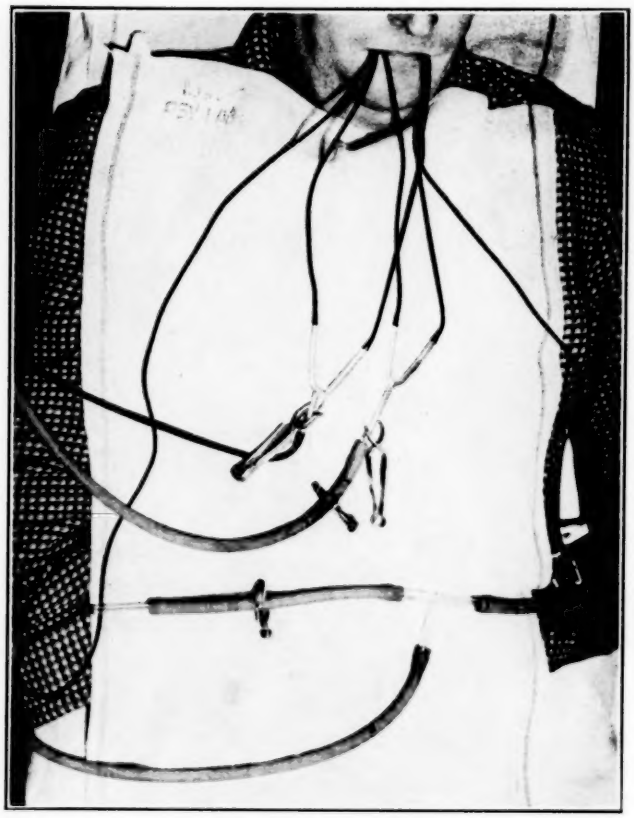

Fig. 5.—Photograph of a subject during an experiment.

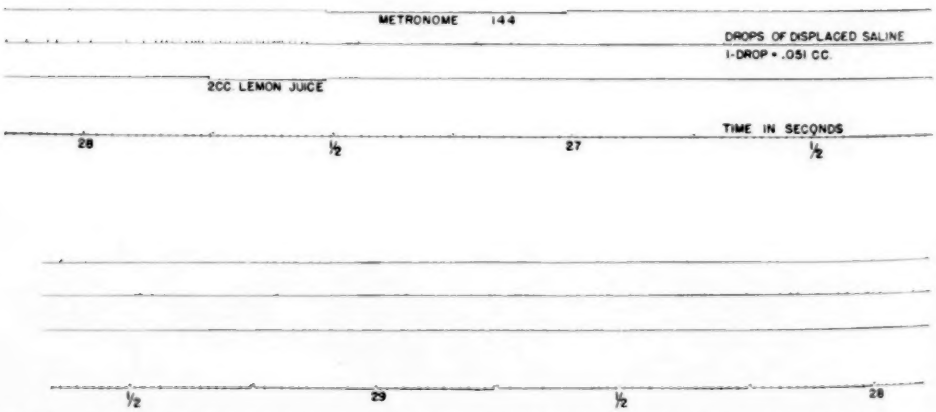

Fig. 6.—Record of salivary response to metronome, 144 beats per minute (conditioned stimulus) and 2 c.c. of lemon juice (unconditioned reenforcing stimulus). To be read from right to left.

previous test. In our procedure the saline system is at the start not connected to the *Y*-tube (Y_1) which leads from the outlet tube of the capsules. Instructions for the procedure are as follows:

1. Close clamp *I*. Open clamp *II*, bringing into the system filling bottle (*F*), which contains a supply of the 4 per cent sodium chloride solution. Open clamps *III* and *IV*. This enables saline to flow through tube (*S*), partly filling reservoir (*R*), but leaving air bubbles at the top of the reservoir, and at the top of the wide part of the long tube (*L*).

2. Close clamp *III*, disconnect reservoir (*R*) from rubber connections at *S*, and pull the short piece of glass tubing (*G*) out of the bent piece of rubber tubing that leads to the capillary dropper (*D*). Tip reservoir (*R*) so that tube (*S*) is lower than *L* blowing into *R* through tube (*G*). The air bubbles in the upper part of *R* will be forced out through *S*. When the saline has filled *S*, close clamp *IV*. An air space remains in the upper part of *L*.

3. Close connections at *S* and *G*, and open clamps *III* and *IV*. Saline again flows into *R* from *F*, and this time displaces the air from *L* and fills the system from *L* to the capillary tip of the dropper (*D*).

4. When saline starts to drop into the buret from *D*, close clamp *III* and open clamp *I*. Saline then flows back past clamp *I*, and out through the end of the rubber tubing at *I*. Close clamps *I* and *II*. From this point on throughout the test clamp *II* remains tightly closed. Any leakage from *F* past this clamp would interfere with the accuracy of the results.

The capsules with the two sets of rubber connections for saliva and for suction and with *Y* tubes Y_1 and Y_2 are sterilized before each test. With Y_2 connected to the suction system, attach a capsule to the inner surface of each cheek so that the inner chamber of the capsule is directly over Stenson's duct. Lemon juice (1 to 2 c.c.) is then injected from the feeding system (see Fig. 4) causing saliva to fill the inner chamber of the capsule, the rubber tubing, and *Y*-tube (Y_1). When saliva starts to drip from Y_1, connect Y_1 to the saline system at *E*, and open clamps *I* and *III*. The system is now completely filled with fluid and ready for the start of the test.

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THE DETERMINATION OF TOTAL FIXED BASE IN TISSUE*

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INTRODUCTION

THE insolubility of benzidine sulfate has been applied to the determination of sulfates in biologic material.[1, 2] Later the method was adapted to the estimation of total fixed base in urine[3] and similar materials.[4] The Stadie-Ross method has been modified by other investigators.[5, 6]

The principle of the method is the conversion of the inorganic salts to sulfates by evaporation of their solution to dryness with an excess of sulfuric acid, followed by ignition, to free the residue of free sulfuric acid. The mixture of sodium, potassium, magnesium, and calcium sulfates thus obtained is taken up in water and the sulfates precipitated with benzidine by adding a known amount of a standard solution of benzidine hydrochloride. The Stadie-Ross procedure is to filter, and titrate an aliquot of filtrate with a standard solution of sodium hydroxide. From this result the amount of total base can be calculated.

In determining the amount of total base in tissue the Stadie-Ross method was chosen as being the simplest for a small amount of material. Van Slyke[7] converted the bases to sulfates and determined the latter as barium sulfate gravimetrically, but the method requires a relatively large amount of sample. It was found that the directions given by Stadie and Ross could not be used for the determination of total base in tissue without considerable modification. This is due chiefly to the small amount of base and the large amount of phosphate present in tissue. The directions for the determination as worked out are given below.

PROCEDURE

Cut the sample of tissue into small pieces and dry to constant weight at 110° C. Digest an accurately weighed portion of about 1 gm. of finely ground dried sample with 1 c.c. of concentrated sulfuric acid and 3 c.c. of 70 per cent perchloric acid. It is desirable to use only a small amount of sulfuric acid as it is to be neutralized later. Heat with a very small flame at first as the perchloric acid is an extremely active oxidizing agent. If overheated at the beginning of the reaction, it may cause combustion or even an explosion. The stock bottle of perchloric acid should not be left under the hood where the digestion is being carried out. Nitric acid may be used, although no difficulty was experienced in using perchloric acid. The latter leaves no ash. The excess

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volatilizes, as perchloric acid is more volatile than sulfuric acid.[8] It may be advisable to cool and add more perchloric acid if the digestion is very slow. The digestion takes some hours.

When the digestion is complete transfer the clear liquid quantitatively to a 50 c.c. Erlenmeyer flask, bringing the volume to about 30 c.c. Add a drop or two of phenol red which will give a pink color in concentrated acid. Neutralize with concentrated ammonium hydroxide. This requires about 3 c.c. The indicator changes to yellow as the acid is diluted, then again to pink at pH 6.8, becoming red if the alkalinity is increased. Make just acid with a few drops of dilute sulfuric acid (1:4). Add 5 c.c. of a 0.1 M solution of ferric ammonium sulfate. A cream colored precipitate of ferric phosphate is produced. The 0.1 M solution of ferric ammonium sulfate will increase the acidity of the solution to approximately pH 3, and the solution of ferric ammonium sulfate hydrolyzes to give less than pH 1. Add several drops of dilute ammonium hydroxide (1:4) to precipitate excess iron as ferric hydroxide. Dilute to 50 c.c. and filter through dry ashless filter paper into a dry flask.

Transfer 10 c.c. portions of filtrate to platinum or silica evaporating dishes, add to each 1 or 2 drops of concentrated sulfuric acid to insure an excess of the sulfate ion, and evaporate to dryness. Great care must be taken to avoid spattering as the amount of liquid becomes small. At this point it is very easy to undergo mechanical loss by spattering and thus introduce a large error. If any spattering is observed a new aliquot should be taken. The method used was to evaporate partially on an electric hot plate at low heat, then to place the dishes on an asbestos mat on the plate and evaporate to complete dryness. Ignite over a free flame, again being careful to avoid overheating at first as ammonium sulfate sometimes decomposes explosively, scattering the contents of the dish. Heavy fumes appear from the decomposition of the ammonium sulfate. Ignite to a bright red heat, so that excess sulfuric acid is decomposed.

Dissolve the ash in water and transfer to a 25 c.c. volumetric flask. It is advisable to add phenol red and titrate with 0.02 N sodium to be sure that no free acid is present. Less than 0.05 c.c. will usually cause the solution to turn pink. Add 1 c.c. of a saturated solution of benzidine hydrochloride and dilute to 25 c.c. Filter through dry ash-free paper into a dry flask. Transfer 20 c.c. of filtrate to a second flask, add phenol red and titrate with 0.02 N sodium hydroxide solution using a microburet. Multiply the result by 5/4, and subtract from the value obtained by titrating 1 c.c. of the benzidine hydrochloride with 0.02 N sodium hydroxide, using phenol red as an indicator. This gives the amount of sulfate in 10 c.c. of the digested liquid, in terms of a known amount of a standard solution of base. From this the amount of base in the sample in terms of milliequivalents can readily be calculated.

DISCUSSION

The method was worked out using dried beef liver. Digestion was carried out in Pyrex Kheldahl flasks, which had been used for some time and were considerably etched. To determine the extent to which the flasks were attacked during the digestion, determinations were made with potassium sulfate, directly

and added to blank determinations on the acids heated in the digestion flasks. The results are given in Table I. A correction of 7 per cent was made on the unknowns.

TABLE I

BLANK DETERMINATIONS ON FLASKS USED
K_2SO_4 IN MILLIEQUIVALENTS

AMOUNT USED		AMOUNT FOUND
0.100	Determined directly	0.0999
0.100	Determined directly	0.0992
0.100	With acid from flasks added	0.108
0.100	With acid from flasks added	0.106
0.100	With acid from flasks added	0.107

When duplicates were found to give reasonable checks with unknown samples, known solutions were made up to see whether the method as worked out was yielding the actual amount of base present. The known solutions were made up to approximate the content of the unknown. The results obtained are given in Table II.

The result obtained with beef liver was 0.0779 m. eq. for a 10 c.c. aliquot, with an average deviation from the mean of ±0.00105 m. eq. or ±1.3 per cent. This amounts to 0.39 m. eq. of total base per gram of dried beef liver. The error with the known solutions was slightly less than with the unknown, the average deviation from the mean being ±1.1 per cent.

TABLE II

TOTAL BASE DETERMINATIONS EXPRESSED IN MILLIEQUIVALENTS

BEEF LIVER		KNOWN SOLUTIONS	
AMT. DETERMINED	CORRECTED	AMT. USED	AMT. FOUND
0.0848	0.0789	0.100	0.0990
0.0844	0.0785	0.100	0.0990
0.0848	0.0789	0.100	0.1015
0.0812	0.0755	0.100	0.1002
0.0825	0.0767	0.100	0.0994
0.0831	0.0773	0.100	0.0970
0.0844	0.0785	0.100	0.0980
0.0848	0.0789	0.100	0.1004
	Av. 0.0779 ± 0.00105		Av. 0.0993 ± 0.00106
	or 0.0779 ± 1.3%		or 0.0993 ± 1.1%

Different organs or kinds of tissue vary in the proportions in which the different basic constituents occur.[9-11] Also the basic content of a particular kind of tissue, as well as the proportion in which the bases occur, varies from one species to another.[12] The test of the method was generalized, therefore, by making up the known solutions to contain equivalent amount of the four bases, sodium, potassium, calcium and magnesium. These solutions were made up to contain 0.025 m. eq. of sodium chloride, 0.025 m. eq. of calcium sulfate in 0.5 N hydrochloric acid, 0.025 m. eq. of magnesium sulfate, and 0.025 m. eq. of potassium sulfate per cubic centimeter, respectively. "Baker's analyzed" salts were used directly.

The data given in Table II under "known solutions" were obtained by transferring 5 c.c. of each of the above salt solutions to a 50 c.c. volumetric

flask, adding 1 c.c. of concentrated sulfuric acid and 2 m. eq. of phosphoric acid. This solution was neutralized, phosphates precipitated and the method continued as described under "procedure." The total base in the 50 c.c. was 0.5 m. eq. Ten cubic centimeters of aliquots were used for evaporation which is the amount referred to in the table. The figures in both columns are arranged so that each two successive sets of figures are from two 10 c.c. aliquots from the same original 50 c.c. solution. This indicates that the largest error of the experimental procedure occurs during or after the evaporation process rather than before, since the error may be small between different 50 c.c. samples, but large between 10 c.c. portions of the same sample. Twenty cubic centimeters portions and 2 c.c. of benzidine hydrochloride were also used, but the error did not seem to be any less and only 2 aliquots are available.

Five cubic centimeters of the iron solution, but not much less, were sufficient to precipitate the phosphorus both in the known and unknown solutions, as shown by tests on the filtrate. Attempts were made to determine the total base in known solutions without precipitating the phosphorus. They were unsuccessful since the fused ash could not be removed quantitatively from the platinum dishes with water. In the absence of phosphates the ash is readily removed.

The acidity of the filtrates after phosphate precipitation, determined with a quinhydrone electrode, varied from pH 3.0 to pH 7.0. The more acid filtrates contain relatively large amounts of iron. On ignition the ferric sulfate is changed to ferric oxide, which is removed with the precipitate of benzidine sulfate. The presence of the iron did not affect the accuracy of the method. Kirjan[6] says that the filtrate should be acidified as calcium and magnesium may be partially converted to the oxides when evaporated in an alkaline medium.

The method is particularly applicable to the determination of total base in the organs of albino rats, or in other cases where the amount of sample available is small.

SUMMARY

The Stadie-Ross method has been applied to the determination of total fixed base in tissue. A relatively small amount of sample is required. The error of the method for 0.1 m. eq. of total base is a little more than ±1 per cent.

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THE TOXICITY OF SILICON*

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THE dictum "Silicosis will not arise in the absence of appropriate exposure to silica (silicon dioxide)" is now generally accepted. Other common silicon compounds, such as ordinary silicates, with the exception of asbestos (a complex magnesium silicate), do not possess the capacity to induce fibrotic changes comparable to those found in silicosis. Silicates other than asbestos, and a great variety of other minerals, under conditions of extensive and prolonged exposure may lead to a moderately increased fibrosis within pulmonary tissues, but such changes do not bear the characteristics of silicosis. When the customary features of silicosis arise after exposure to such substances, free silica may be shown to be present as an impurity or admixant. Apart from the peculiar fibrogenetic properties possessed by silica, it appears possible from recent investigations that some compounds of silicon may possess toxic properties in no known respect related to the fibrogenetic action of silica. This statement is with special reference to organic compounds of silicon, such as ethyl and methyl silicates, which substances are now finding industrial application. Such quantities as 0.25 ml. of ethyl silicate per kilo are lethal for rabbits within one minute. In the midst of an investigation directed to the toxicity of organic silicon compounds, it became desirable to ascertain if elemental silicon itself possesses demonstrable damaging properties. This brief report is based upon limited animal experiments, in which highly purified silicon† was administered to animals through various methods.

In these experiments, 44 animals (guinea pigs and albino rats) were utilized. Varying quantities of silicon (0.1 or 0.2 gm. per animal) in particle size below 10 microns, suspended in distilled water, were administered intraperitoneally, subcutaneously, or intramuscularly. Chief interest centers about those animals injected intraperitoneally. On the second, fourth, seventh, twelfth, nineteenth, thirtieth, and sixtieth days, some experimental animals, together with their controls, were killed for gross and microscopic examination. The general procedure followed was that first introduced by Miller and Sayers[1] and later described by McCord[2] and others.

Following intraperitoneal injections, utilizing the procedures to be found in the publications just cited, responses from various types of dusts fall into three main categories, namely (1) the proliferative, which is characteristic of the action of silica in its various forms, such as quartz, tripoli, amorphous, etc.; (2) inert, which is characteristic of such substances as iron oxide, titanium

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†The crystalline silicon employed was obtained from the Electro-Metallurgical Company, New York City, and was certified as representing a purity of 99.85 per cent elemental silicon.

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dioxide, carbon, etc.; and (3) absorptive, which is characteristic of limestone, calcite, gypsum, etc. Those substances which in human beings, after due exposure, lead to clinical silicosis, invariably, under appropriate experimental procedure lead to proliferative reaction within the peritoneal confines. Conversely, those dusty materials to which workers may be exposed almost indefinitely with relative impunity, so far as fibrogenetic changes are concerned, yield either inert or absorptive manifestations. These changes become apparent within thirty days and no remarkable deviations arise after sixty days following intraperitoneal injections. The inference apparently warranted from the use of this measure of physiologic responses on the part of animals is that in the absence of proliferative changes under experimental conditions properly carried out, any dust of unknown constituency may be regarded as substantially harmless in its capacity to induce pulmonary fibrotic changes among exposed human beings.

Introducing elemental silicon by subcutaneous, intramuscular, and peritoneal methods, observations were made both for evidences of immediate toxicity, such as are associated with organic silicon compounds and the cellular changes of any of the three types described by Miller and Sayers. Invariably, no quick damage was detected other than that of minor trauma and apart from foreign body reactions, the changes induced over long periods of time were routinely typical of the Miller and Sayers "Inert reaction."

Within the limits of conclusiveness afforded by this type of experiment, the opinion is formed that of the known injurious properties possessed by divers types of silicon compounds, none are known to be possessed by elemental silicon.

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LABORATORY METHODS

VALUE OF THE HAGEDORN-JENSEN METHOD FOR DETERMINING PENTOSES AND OTHER REDUCING SUGARS FORMED BY HYDROLYSIS OF POLYSACCHARIDES*

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PREVIOUS studies of the hydrolysis of polysaccharides obtained from cultures of tubercle bacilli, using the Shaffer-Hartmann[1] method for the quantitative determination of the carbohydrates, have been reported by Seibert and Munday,[2] Renfrew,[3] Masucci, McAlpine and Glenn,[4, 5] and Anderson and Roberts.[6] Renfrew[3] reported that by heating the carbohydrate with 5 per cent sulfuric acid for eight hours in a boiling water-bath the reducing properties were greatly increased. Seibert and Munday[2] also found that, unless previously hydrolyzed, the amount of reducing substances was very small, and that a seven-hour period of acid hydrolysis in a water-bath was the proper time interval for hydrolysis, at which time it was complete. Seibert and Munday[2] state that if the hydrolysis was continued longer than seven hours, there was a small decrease in the percentage of the reducing substance. Munday and Seibert[7] found the Hagedorn-Jensen method[8] to be more sensitive for the determinations of practically all reducing substances.

The present study was undertaken to determine the optimum time for hydrolysis of the polysaccharides, using the Hagedorn-Jensen ferricyanide method. Especial attention was given to the carbohydrates of the five carbon groups, since according to Anderson and Roberts[6] and Masucci, McAlpine and Glenn[4] a considerable portion of the polysaccharides obtained from cultures of tubercle bacilli are pentoses.

EXPERIMENTAL

Special care was taken in selecting pure substances for the determinations. The polysaccharide fraction of a human strain of tubercle bacilli used in the experiment was donated to us by Dr. Florence B. Seibert, and was known to be a crude product.

A small amount of the carbohydrate was accurately weighed and dissolved in 250 ml. of distilled water. To ten different aliquots of 15.625 ml. each of this solution, 0.5 ml. of concentrated sulfuric acid was added. The tubes containing the samples were attached to "reflux" condensers and placed in a large receptacle of boiling water. Two tubes were withdrawn from the condensers at each of

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the different time intervals. The carbohydrate solution was then neutralized with NaOH and made up to 50 ml. From the neutral solution made from the contents of each tube, two samples of 1 ml. each were used for the sugar determinations. In no case did the sample contain more than 0.3 mg. of carbohydrate, as is required for the Hagedorn-Jensen method. The analysis was conducted as follows: To 1 ml. of the solution 11 ml. of distilled water were added. To this, 2 ml. of the Hagedorn-Jensen ferricyanide reagent* were added and the solution boiled for exactly fifteen minutes in a water-bath. After cooling, 3 ml. of a freshly prepared KI-$ZnSO_4$-NaCl reagent was added.

Then 2 ml. of a 3 per cent acetic acid solution and 0.5 ml. of a 1 per cent starch solution were added. Titration was done with 0.002 N/$Na_2S_2O_3$. Blanks were run at the same time.

TABLE I

SUBSTANCE	HOURS HYDROLYZED	MG. SUBSTANCE TESTED	C.C. .002N $Na_2S_2O_3$ EQUIVALENT TO SAMPLE	C.C. N $Na_2S_2O_3$ EQUIVALENT TO 1 GM. SUBSTANCE	GLUCOSE EQUIVALENT %
Glucose	0	0.222	3.1	27.92	100
	1	0.237	3.45	29.08	104.15
	3	0.237	3.52	29.67	106.26
	5	0.237	3.48	29.33	105.05
	7	0.237	3.40	28.66	102.65
	10	0.237	3.16	26.63	95.37
Mannose	1	0.246	3.63	29.48	105.58
	3	0.246	3.48	28.26	101.21
	5	0.246	3.55	28.82	102.86
	7	0.246	3.52	28.58	102.36
	10	0.246	3.36	27.28	97.70
Maltose	1	0.196	2.58	26.21	93.75
	3	0.196	2.77	28.15	100.82
	5	0.196	2.77	28.15	100.82
	7	0.196	2.79	28.35	101.54
	10	0.196	2.67	27.13	97.17
Arabinose	1	0.171	2.28	26.61	95.30
	3	0.171	2.20	25.69	92.01
	5	0.171	2.27	26.50	94.91
	7	0.171	2.07	24.17	86.56
	10	0.171	2.06	24.05	86.13
Xylose	1	0.167	2.33	27.82	99.64
	3	0.167	2.27	27.10	97.06
	5	0.167	2.23	26.62	95.34
	7	0.167	2.18	26.02	93.19
	10	0.167	2.02	24.11	86.35
Gum acacia	1	0.177	1.42	16.02	57.37
	3	0.177	1.83	20.65	73.96
	5	0.177	1.91	21.55	77.18
	7	0.177	1.86	20.99	75.17
	10	0.177	1.87	21.10	75.93
Polysaccharide Fraction TB 119 (human)	1	0.47	1.62	6.89	24.67
	3	0.47	2.18	9.27	33.20
	5	0.47	2.28	9.70	34.74
	7	0.47	2.30	9.78	35.02
	10	0.47	2.03	8.63	30.90

*The Hagedorn-Jensen reagent was prepared by dissolving 0.825 gm. of $K_3Fe(CN)_6$ and 5.3 gm. of Na_2CO_3 (ignited) in water and making the solution up to 500 c.c. The KI-$ZnSO_4$-NaCl reagent was prepared by dissolving 5, 10 and 50 gm., respectively, of these substances in water and making the solution up to 200 c.c.

Calculations for glucose were made according to the following formula: Number of cubic centimeters of $0.002N/Na_2S_2O_3$ used for the blank, minus number of cubic centimeters of $0.002N/Na_2S_2O_3$ used for the unknown divided by 2.5 = K. The amount of glucose in milligrams was computed by solving the formula:

$$0.1735\ K + \frac{0.005K}{2.27-K} = \text{milligrams of carbohydrate in the solution calculated as glucose.}$$

In Table I are given the results of the analyses for the different substances used: Glucose, mannose, maltose, arabinose, xylose, gum acacia, and the polysaccharide fraction obtained from the filtrate of a culture of a human strain of tubercle bacilli.

COMMENTS

In all cases, prolonging the hydrolysis of the carbohydrates more than seven hours results in the destruction of some of the reducing substances, as previously found by Seibert and Munday[2] for polysaccharides prepared from tuberculins. The decomposition of pure monosaccharides begins after the five-hour interval of hydrolysis, and the loss due to continued heating at the temperature of boiling water is more marked in some carbohydrates than in others. In the present analyses the fall in the percentages calculated as glucose, for glucose, arabinose and xylose after five hours heating are 2.4 per cent, 8.35 per cent, and 2.15 per cent, respectively; while the loss for mannose after the same period of hydrolysis is only 0.5 per cent.

Heating polysaccharides during acid hydrolysis for a period of more than five hours undoubtedly results in the decomposition of some of the hydrolysis products, and if arabinose is one of the resulting substances the loss is very considerable. The pentose-yielding polysaccharides are more labile to this decomposition of the reducing substances, as is demonstrated by the analysis of gum acacia. Apparently, the loss in the percentages of the reducing substances of the polysaccharides is not so great as would be expected, probably because it is some time before the lower form carbohydrates are released and so exposed to the action of the heat.

The breaking down of the hexose molecule occurs during the acid hydrolysis and at the same time that hexose is being freed from polysaccharide; so that the final result is a balance between these two processes, and whichever occurs faster determines the result. The decomposition in the extended ten-hour period of hydrolysis is so great that the products have lost the ability to reduce.

SUMMARY

1. Monosaccharides subjected to acid hydrolysis for a period of more than five hours undergo partial decomposition. This loss is greater for some substances than others; pentoses, like arabinose, undergo a considerable destruction.

2. Prolonging the hydrolysis of polysaccharides more than seven hours results in the destruction of some of the reducing substances released during the reaction, as previously demonstrated by Seibert and Munday[2] using the Shaffer-Hartmann method for the sugar determinations.

3. A hydrolysis of more than five hours for pentose-yielding polysaccharides may result in the destruction of some of the hydrolyzed products.

4. The Hagedorn-Jensen method is very satisfactory for determining substances of low reducing power as indicated by Munday and Seibert.[7]

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A SIMPLE DIRECT READING PHOTOELECTRIC COLORIMETER*

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THE eye is not an accurate optical matching instrument. Even selected observers, who have normal chromatic perception, vary from time to time and differ with each other on the same set of colors. Systemic fatigue, as well as eye fatigue itself, intoxications, and even minor pathologic states will cause considerable discrepancies in observations.[1] The photoelectric cell offers an accurate, objective and convenient means of measuring light intensities.

REVIEW OF THE LITERATURE

Photoelectric colorimeters fall into two general categories. The simplest in design and operation is the direct reading type. This consists essentially of a single photocell, a light source, and a suitable container for holding the test solution between the light source and the sensitive surface of the photocell. After its passage through the test solution, the light falls on the sensitive surface of the photocell generating a minute electrical current which is read directly on a microammeter. The logarithm of the amperage generated by the beam of light after traversing the test solution bears a linear relationship to the concentration of the test solution. Reimann,[2] followed by Armstrong and Kuder,[15] Sanford and others,[3] Richards and John,[4] Ellis,[5] Diller,[6] Evelyn,[7] and others, was the first to devise this type of instrument. The limits of measurement of the apparatus are restricted to the range between the maximum and minimum outputs of the cell. This range is relatively small and would be a serious drawback in some fields, but is ample and satisfactory for clinical pathological determinations. The chief trouble with this colorimeter has been to obtain a stable, fixed light source since fluctuations in the illuminant produce considerable variations in the output of the photocell. Due to this difficulty Lange,[8] Exton,[9] Shook and Scrivener,[10] Goudsmit and Summerson[11] among others developed a double photocell null point type whose operation is independent of fluctuations of the light source. Here two identical photocells are placed on the same optical axis with the light source between them and appropriate containers for test solutions on either side of the illuminant. The light is movable and in practice is adjusted so that the outputs of the two cells are balanced against each other through a Wheatstone bridge at zero reading of a galvanometer. The concentration of the test solution stands in immediate relationship to the scale reading of the light source and not the output of the cells. In Exton's[9] instrument, there is in addition a calibrated, variable aperture between one photocell and the lamp, and in certain types

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of measurements the values of the test solutions are a function of the aperture scale reading at which the outputs of the cells balance. This apparatus is more complex in design and operation than the post type. Besides, if photoemissive cells are used, as is the case in a number of designs,[9] fluctuations in the voltage of the "B" battery or "B" battery eliminator will alter the flow of current through the cells and hence the output of the cell. Furthermore, it is difficult to get a pair of cells that respond identically.

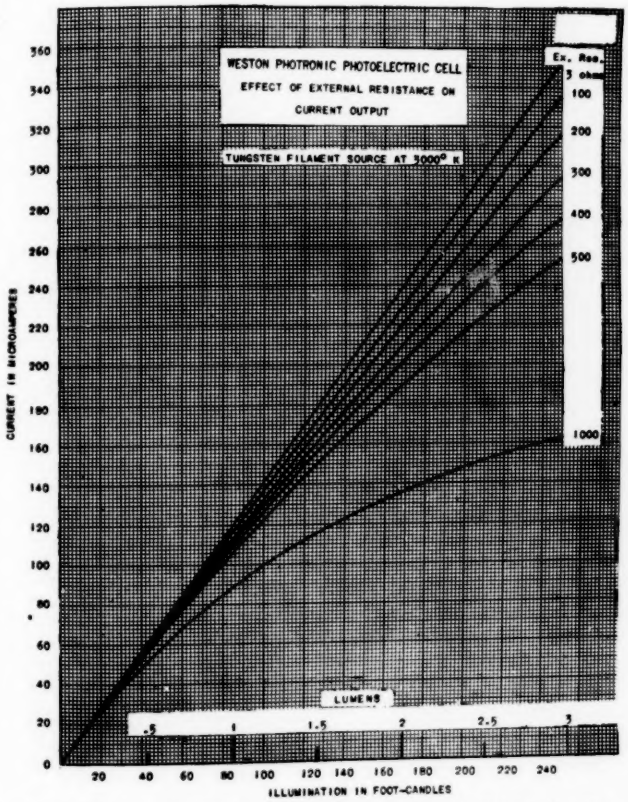

Fig. 1.—Output of the Weston photronic cell in relation to the light intensity and the external resistance. (Courtesy of Mr. W. N. Goodwin, Weston Electrical Instrument Corp.)

PHYSICAL CONSIDERATIONS

A photocell for direct reading photoelectric colorimetry should possess the following characteristics: (1) A relatively high electrical output per lumen or foot-candles; (2) a constant electrical output under a given illumination; (3) a color sensitivity curve of approximately the same range as the human eye, and (4) simplicity of operation, ruggedness of construction, and the ability to withstand excessive illumination even when short circuited.

The Weston photronic cell, model 594, satisfactorily meets these requirements. It is dry photovoltaic cell of the "barrier plane" ("Sperrshicht") type consisting essentially of a thin iron disc on which is sputtered a film of selenium, the light sensitive element. The disc forms the positive pole while

a metal collector ring in contact with the light sensitive film is the negative pole. Its output per foot-candle and per lumen is high, being 1.4 and 120

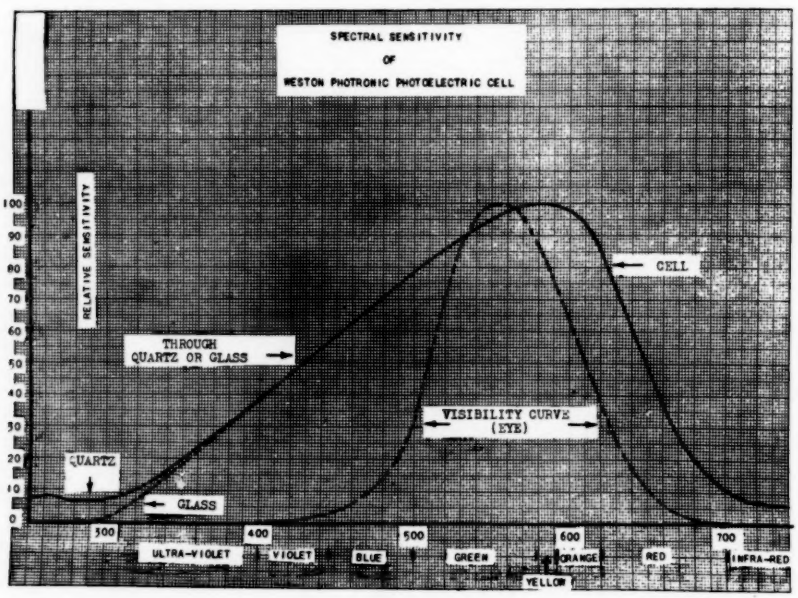

Fig. 2.—Comparison of the spectral sensitivity range of the Weston Photronic cell with the visibility curve of the human eye. (Courtesy of Mr. W. N. Goodwin, Weston Electrical Instrument Corp.)

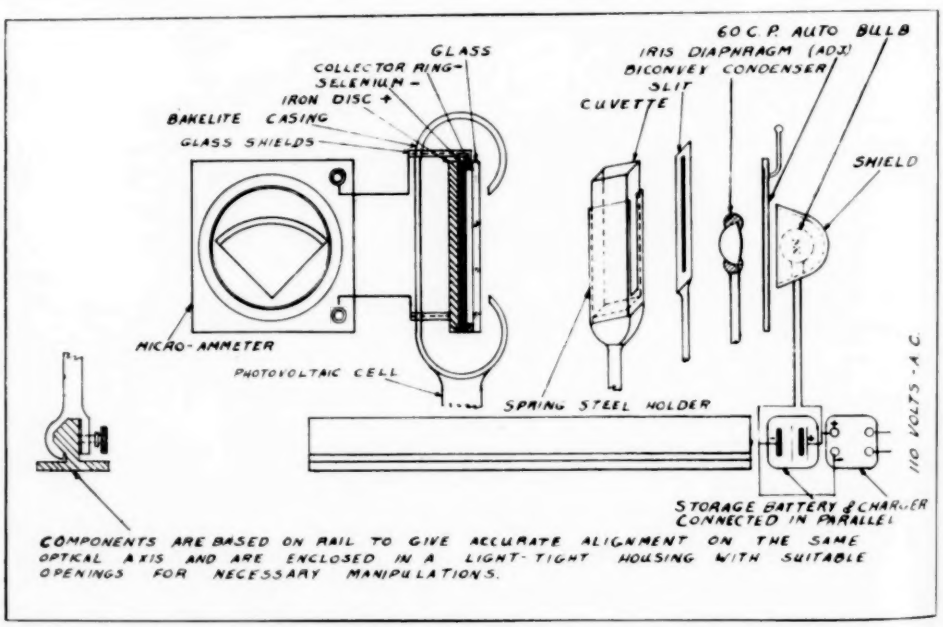

Fig. 3.—Diagram of the colorimeter.

microamperes, respectively.[12] Hence the current can be read directly on a sensitive ammeter without the need of amplification. This makes for simplicity

TABLE I

DEXTROSE DETERMINATION. FOLIN-WU TECHNIQUE. AQUEOUS DEXTROSE SOLUTION CONTAINING 0.1 MG. PER C.C.

NO.	CUBIC CENTIMETER OF DEXTROSE SOLUTION	EQUIVALENT TO CONCENT. OF MILLIGRAMS PER 100 CUBIC CENTIMETERS	READING OF AMMETER IN MICROAMPERES
1	0.50	50	71.4
2	0.70	70	58.4
3	0.90	90	53.8
4	1.10	110	47.4
5	1.30	130	40.3
6	1.50	150	35.1
7	1.70	170	29.4
8	1.90	190	25.5
9	2.10	210	23.0
10	2.30	230	20.8
11	2.50	250	18.9
12	2.70	270	16.6
13	2.90	290	15.0
14	3.10	310	14.3
15	3.30	330	11.8
16	3.50	350	11.0
17	3.70	370	10.0
18	3.90	390	9.8
19	4.10	410	8.2

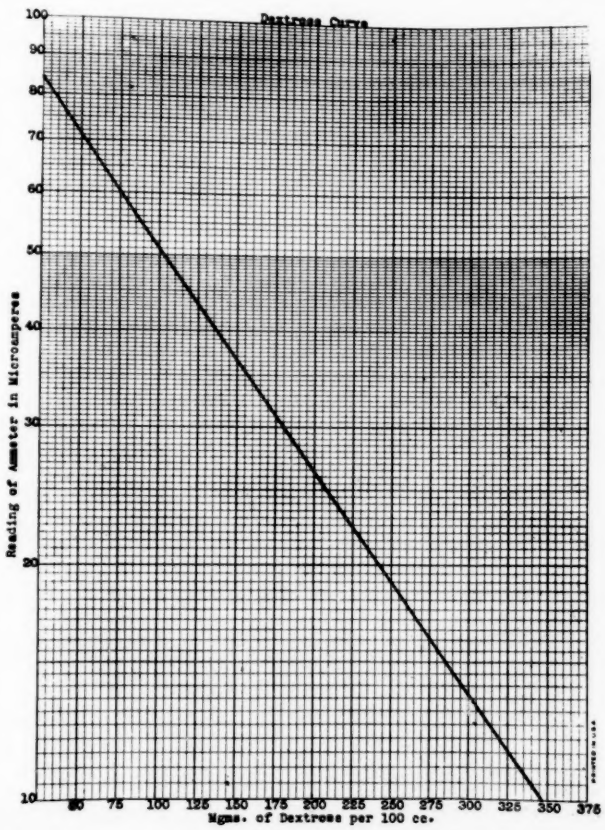

Fig. 4.—Dextrose calibration curve.

TABLE II

CREATININE DETERMINATION. ALKALINE PICRATE TECHNIQUE. AQUEOUS CREATININE SOLUTION CONTAINING 0.1 MG. PER C.C.

NO.	CUBIC CENTIMETERS OF CREATININE SOLUTION	CUBIC CENTIMETERS OF WATER	EQUIVALENT TO CONCENT. OF MILLIGRAMS PER 100 CUBIC CENTIMETERS	READING OF AMMETER IN MICROAMPERES
1	1.00	9.00	1.0	82.3
2	1.50	8.50	1.5	81.0
3	2.00	8.00	2.0	79.0
4	2.50	7.50	2.5	78.0
5	3.00	7.00	3.0	77.6
6	3.50	6.50	3.5	75.7
7	4.00	6.00	4.0	74.0

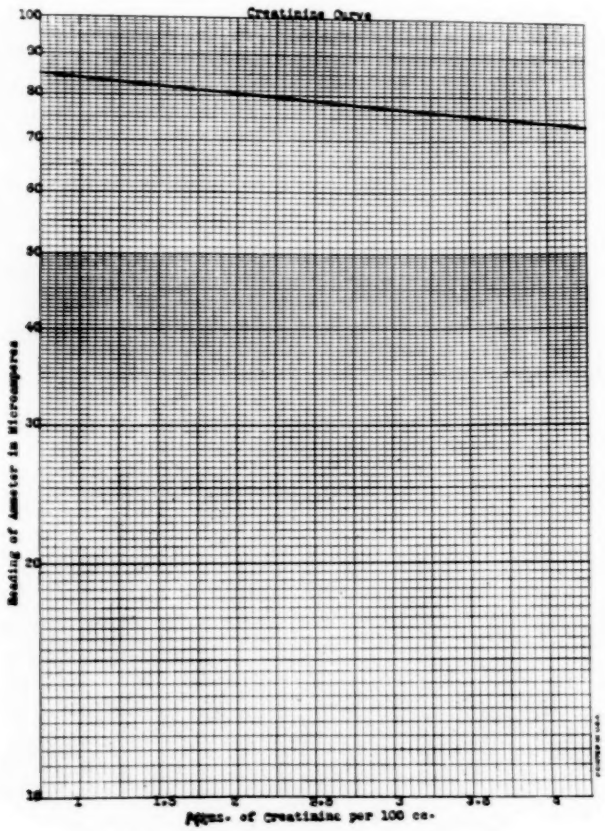

Fig. 5.—Creatinine calibration curve.

of operation and, furthermore, any errors inherent in the apparatus are not magnified as they would be by an amplifier. Its output is constant for a given illumination (Fig. 1), but it has a property called "fatigue." On first exposure to light, the output of the cell gradually falls, becoming constant in a few minutes. In practice the cell must be exposed to light for about ten minutes and "fatigued" to a constant output before the colorimeter can be used. The biologic application of this instrument requires that its light sensitive element have a color sensitivity closely approximating that of the human

TABLE III

URIC ACID DETERMINATION. NaCN—ARSENIC PHOSPHOTUNGSTATE TECHNIQUE. AQUEOUS URIC ACID SOLUTION CONTAINING 0.02 MG. PER C.C.

NO.	CUBIC CENTIMETERS OF URIC ACID SOLUTION	CUBIC CENTIMETERS OF WATER	EQUIVALENT TO CONCENT. OF MILLIGRAMS PER 100 CUBIC CENTIMETERS	READING OF AMMETER IN MICROAMPERES
1	1.00	9.00	2	28.8
2	1.50	8.50	3	18.6
3	2.00	8.00	4	13.0
4	2.50	7.50	5	10.4
5	3.00	7.00	6	7.8
6	3.50	6.50	7	5.9

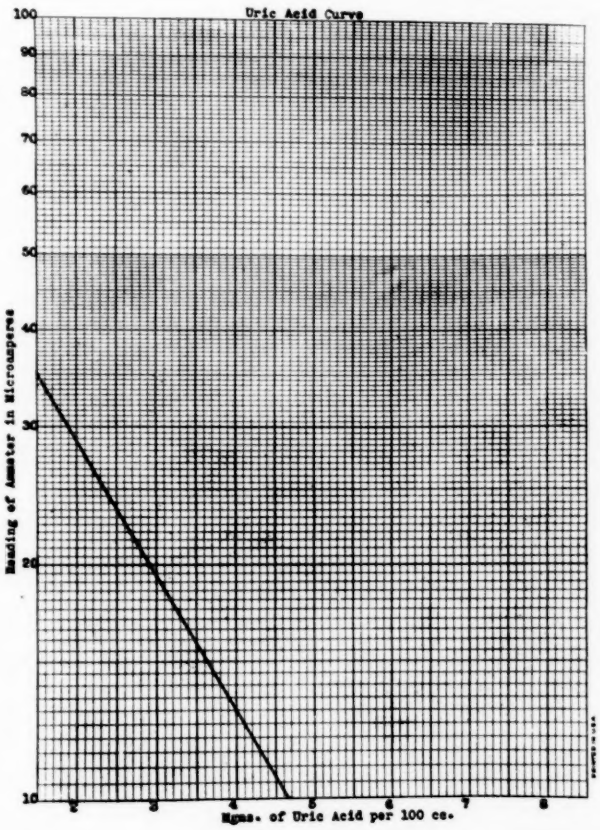

Fig. 6.—Uric acid calibration curve.

eye. The photronic cell meets this requirement quite closely (Fig. 2). Furthermore, the photronic cell requires no "A" or "B" batteries, its operation depending entirely on the electronic action of light on the sensitive surface.[13] It is compact, rugged, and cannot be harmed by exposure to excessive light.

According to Beer's law the absorption of light passing through a solution depends on the concentration and thickness of the layer of solution traversed. Where I_o = the intensity of the incident beam of light, I = the intensity of the exit beam of light, a = the absorption coefficient, d = the thickness of

the layer of solution traversed, and c = the concentration of the solution

$$(1)\ I = I_o a^{dc} \quad (2)\ dc \log a = \log I_o - \log I \quad (3)\ \therefore c = \frac{\log I_o - \log I}{d \log a}$$

The photocell measures "I" in amperes. The value of "a" is determined experimentally while "I_o" and "d" are kept constant by fixing the light source and using the same container in the same position, respectively. Hence the log of "I" in amperes plotted against "c" is a straight line provided the external resistance of the cell is low enough (Figs. 1, 4, 5, and 6).

EXPERIMENTAL

As indicated before, a reliable direct reading photoelectric colorimeter must possess a light source of absolute stability. Any given change in the line voltage supplying the light source results in a five-fold change in the microamperage output of the photocell. Different trials with various schemes to maintain a constant voltage led to the adoption of a battery system, there being no simple, inexpensive voltage regulating device available.[14] The voltage and amperage of a fully charged storage battery are practically constant, falling off only gradually with the loss of its charge. The disadvantages of storage batteries are clearly obvious, the chief one being the necessity of keeping the battery fully charged. This inherent difficulty is largely overcome by connecting a small automobile battery charger in parallel with the battery (Fig. 3). The battery is first fully charged and with the colorimeter in operation, the charger is simply connected to the power line. In effect practically all of the current is drawn directly from the power line, the battery steadying any fluctuations in the line by absorbing any rises in the output and throwing energy back into the circuit with a fall in output. Therefore, the battery serves merely as a "fly wheel" and not as a direct source of energy. This arrangement was checked by placing a millivoltmeter across the terminals of the illuminant lamp (Fig. 3). Variations of not more than 2 or 3 millivolts were found in the lamp circuit. The maximum fluctuation in the current of the photocell with this hook-up under fixed conditions is not more than 1 microampere. To preclude the possibility of upsetting the stability of the light source all variations in the intensity of the light source are controlled by an iris diaphragm (Fig. 3).

APPARATUS

The apparatus consists of a photocell, cuvette container for solutions, slit, condenser, iris diaphragm, and light source in the order named (Fig. 3). All of these are mounted on a heavy track, triangular in cross-section to keep them in the same optical axis while permitting them to be moved individually or collectively. Their positions on the track can be fixed by a thumb screw. The photocell is a Weston photronic cell, No. 594, connected to a Rawson quadruple range double pivot ammeter, type 501, which reads 100, 10, 1.0, and 0.1 amperes on its four different ranges when the pointer makes a full excursion over 100 scale divisions. The 0.1 ampere range is used, the ammeter having a resistance of 50 ohms, low enough to give a curve of the amperage output of the photocell that is practically a straight line in relation to the

light intensity (Fig. 1). In front of the photocell is a carrier to accommodate an oblong cuvette 7.2 by 1.5 by 1.5 cm. and holding 16 c.c. Two of the parallel sides of the cuvette are frosted while the other two are transparent. The carrier is so constructed that the cuvette can be placed in it in only one way so that the transparent sides and the same depth of solution always face the light beam and the photocell. The beam of light is focused on the cuvette by a slit and a single biconvex condenser placed between the cuvette and the light source. The light source is fixed electrically but to adjust the intensity of the incident beam of light falling on the cuvette an iris diaphragm is fitted between the condenser and the illuminant. The light source is a 60 candlepower Mazda automobile headlight lamp shielded from behind and supplied by a 6 volt automobile storage battery connected in parallel to a small automobile type battery charger.

All electrical connections are carefully soldered and insulated except where binding posts are fitted. To prevent electrical leaks across the terminals of the photocell the first portions of the wires connecting it to the ammeter are enclosed in glass sleeves.

All components of the instrument are enclosed in a light tight housing with suitable openings for necessary manipulations.

APPLICATION

Before the colorimeter can be used calibration curves of electrical values of the exit beam of light *(I)* corresponding to known concentrations *(c)* of the substance to be tested must be plotted (vide supra). The concentration of an unknown can be read at a glance by referring its electrical value to the appropriate graph. To secure these values the cuvette is first filled with distilled water and placed in the carrier. The photocell is "fatigued" in about 10-15 minutes to a constant output and the diaphragm is then adjusted so that the ammeter reads 100 microamperes. The cuvette is emptied and now filled with solutions of known concentration of the test substance and the corresponding electrical values are read off the ammeter.

To operate, fill the cuvette with distilled water, "fatigue" the cell to a constant output, and adjust its output to 100 microamperes with the diaphragm. Replace the distilled water with the test solution, find its electrical value, and read its corresponding concentration from the proper graph.

DISCUSSION

The inherent error of the instrument is about 1 or 2 per cent, due chiefly to changes in the intensity of the light source. The manufacturer states that the output of the cell is constant for a given illumination, but does not indicate the limits of this constancy.

There is no appreciable drift in the calibration curves over a period of at least one year according to Armstrong and Kuder.[15]

The colorimeter in its present design has one serious drawback. With the increase in the concentration of the test solution, there is a decrease in the amperage of the cell. Thus, in the higher concentrations, electrical values

become so small that they cannot be detected even by a very sensitive ammeter, a factor which limits the range of the instrument. For example, the colorimeter is not accurate in dextrose solutions prepared by the Folin-Wu technique in concentrations of over 400 mg. per 100 c.c. (see Table I), but the scope of the instrument is adequate for its primary purpose, namely blood chemistry

This colorimeter is intended primarily to test solutions usually matched with the human eye. The photronic cell has a color sensitivity curve closely approximating that of the human eye (Fig. 2) and measures colors over this scale efficiently. The use of color filters would increase the accuracy of the instrument, but it is accurate between 1 and 2 per cent, without filters and any additional accuracy would not be worth practically the complication of color filters. Furthermore filters would diminish the range of the instrument by rapidly increasing the rate of light absorption, especially in the higher concentrations. As long as the colorimeter gives a constant and reliable relationship between the concentration of a solution and its photoelectrical value, the instrument is sufficiently accurate for ordinary clinical purposes without filters.

SUMMARY

The design, operation, manipulation and some of the uses of a simple, direct reading photoelectric colorimeter are described.

The authors wish to express their appreciation to Drs. Dushman and Koller of the General Electric Research Laboratory in Schenectady, N. Y., for their advice and counsel.

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A NEW METHOD OF STAINING SPIROCHETES AND BACTERIA IN SMEARS*

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IN TISSUE sections prepared by silver salt reduction methods, all *tissue* elements receive silver coats different from those of *spirochetes* or *bacteria* in the same sections: spirochetes are deep black, while tissue structures are light yellow to dark brown. "In tissue sections the new phase Ag does not react monodispersely, but polydispersely with different colloidal distributions."[1] On the spirochetal surface appears a black, opaque, microscopically homogeneous coat of silver, like the back of a silvered mirror. If this coating on spirochetes is indeed like the mirror-back deposit of silver, *the chemical processes used in technical production of silvered mirrors might be useful for the staining of spirochetes also.* In this regard, one may consult the *Handbuch der biochemischen Arbeitsmethoden* of Abderhalden,[2] in which a formula for production of excellent silver mirrors on glass surfaces (test tubes, watch glasses, dishes, etc.) is given. In this method two silver-salt solutions are mixed: a solution of ammoniacal silver nitrate (1 per cent), and a 0.2 per cent silver nitrate solution to which has been added sodium potassium tartrate.

We have tried this method of silver-mirror deposition for the silver-staining spirochetes and bacteria in smears. At first precipitation of silver interfered, but we found this could be entirely avoided by the addition of a protective colloidal substance, gum arabic solution. Fresh smears dried in the air or by warming, and others which had been kept a long time in 10 per cent formalin were used. In the formalin-fixed smears, red blood cells appeared brown after staining; the poor contrast between this color and the black spirochetes made the latter rather difficult to find. For this reason, a different preliminary preparation of the smear was sought. The use of uranium nitrate was tried, since according to Strempel and Armuzzi,[3] Kufs,[4] the treatment of sections with uranium nitrate increases and accelerates the silver reduction and gives a vivid black silvering of the surface of spirochetes. A 2 per cent solution of uranium nitrate in distilled water acting for two or three minutes produces this desirable effect, whereas on the specimens not so treated, silver mirror deposition goes on slowly and at first incompletely. Even in smears which have been preserved dry for two and one-half years without any sort of preparation, spirochetes can be stained very well by preliminary treatment with 2 per cent uranium nitrate solution.

With this method, good results were obtained on smears of blood containing spirochetes of relapsing fever or sodoku (ratbite fever), on smears of serous fluid from human primary syphilitic lesions, and on smears of rabbit testicles and human paretic brains containing *Spirocheta pallida.* However,

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the color contrast between the hemoglobin-bearing brown erythrocytes and the black spirochetes was not sufficiently great to permit the easy discovery of microbes in smears containing only a few organisms. We therefore tried another method of preliminary treatment of the smears. In place of the uranium nitrate we used ammonium hydroxide (10 drops or 1 c.c. of concentrated NH_4OH in 60 c.c. distilled water) and this proved to be satisfactory. Thus, a convenient and reliable method was evolved, which should be useful in both theoretical and practical work. The procedure recommended is as follows:

SILVER MIRROR METHOD

Preparation of Solutions.—Solution A: Ten grams of silver nitrate ($AgNO_3$ cryst. for analysis) are dissolved in 200 c.c. of distilled water; then concentrated NH_4OH is added cautiously until the precipitate of silver oxide which forms is all dissolved but a faint cloud. The solution is then filtered and diluted with distilled water to 1,000 c.c.

Solution B: Two grams of silver nitrate ($AgNO_3$ cryst. for analysis) are dissolved in 1,000 c.c. of *boiling* distilled water, then 1.65 gm. of finely powdered sodium potassium tartrate added and the whole boiled shortly until the white precipitate changes to gray, then filtered while hot.

These solutions are kept separate in brown or amber bottles, where they do not deteriorate for months.

Gum arabic solution: Twenty-five grams of gum arabic are dissolved in 100 c.c. of hot distilled water, sterilized and kept in the ice box.

Staining Procedures.—a. *For Blood Smears:* 1. Immerse warmed or air-dried *well-fixed* smears in dilute NH_4OH (10 drops or 1 c.c. concentrated NH_4OH to 60 c.c. distilled water) in a dish two or three times, until the red color of the hemoglobin disappears. Use caution, because the ammonia may dissolve the smears from the glass surfaces if they have not been sufficiently fixed.

2. Wash thoroughly two or more times in distilled water and keep the slides wet on a staining rack.

3. Mix 10 c.c. of Solution A, 15 c.c. of Solution B, and 18 drops or 1 c.c. of the gum arabic solution. It is advisable to mix these reagents in a graduated cylinder and to shake the mixture thoroughly. The cylinder must be rinsed immediately to avoid deposition of silver on the inner surface. If the silver mirror production goes on too fast, add more gum arabic, up to 2 c.c. total.

4. Spread the mixed solutions over the surface of the slide so that the smear and its surroundings are entirely covered. A series of slides may be stained with one mixture.

5. Allow to stand forty to sixty minutes or longer; one and one-half hours is not too long. If faster deposition is desired, the slides may be heated *almost* to steaming with a Bunsen or alcohol flame. On the unsmeared surface of the slide a smooth silver mirror should appear.

6. Pour off the mixture and wash in distilled water; dry in air.

b. *For Bacterial or Spirochetal Smears or Thick Films Other Than Blood:* The procedure of a, 1 with ammonia water may dissolve smears or thick films

if they are fresh or insufficiently fixed. Even washing with distilled water may do this. With blood smears it is less to be feared because blood and serum adhere well to glass surfaces. To avoid solution of the smears a 2 per cent solution of uranium nitrate in distilled water is used as a fixative for one to two minutes, instead of ammonia as in a, 1. For staining *Spirocheta pallida*, the uranium nitrate solution is first used, the slide washed and *then* immersed in ammonia as in a, 1.

1. Place 2 per cent solution of uranium nitrate over the surface of the slide so that the smear and its surroundings are entirely covered, and let stand for two minutes.

2. Wash thoroughly twice in distilled water, and keep the slide wet.

3 to 6 the same as in a.

Method b has been used on smears and thick films for bacteria of various kinds, on smears from primary and congenital syphilitic lesions of humans and experimental syphilitic lesions of rabbits, and on smears from general paretic brains, with good success in all.

These methods are extremely simple and easy to handle even for an operator not familiar with silver staining. The solutions may, of course, be made up and mixed in half-quantities if desired.

COMMENTS

Spirochetes and bacteria, as well as nuclei of leucocytes, appear deep black; red blood cells are light yellow or colorless; and protozoa, such as trypanosomes and malarial plasmodia, do not take the silver coating. *Treponema pallidum* needs more time (one hour) for deep black silver-staining than other kinds of spirochetes; this may be very clearly seen in smears of serous fluid from primary syphilis contaminated with *Sp. refringens* or other types of spirochetes and bacteria. When the other spirochetes are deep black in color, *Sp. pallida* are dark brown. Bacteria take the silver mirror faster than spirochetes, and for them the time of Procedure 5 can be shortened from five to twenty minutes.

As an interesting fact it may be noted that the process of silver-staining spirochetes goes on unevenly at first. If Procedure 5 is interrupted, the individual spirochetes show nearly equal silver-free intervals, appearing as broken wavy lines ("septierte Formen").

The fixative and intensifying action of uranium nitrate is complicated. Its effect depends upon the concentration of the salt, the time and temperature of procedure and the amount of material being fixed. If uranium nitrate is used too long and in too high a concentration, its effect is reversed: an incomplete and slow deposition of silver occurs. Another surprising effect is seen with prolonged use of highly concentrated uranium nitrate solution, or with a mixture of equal parts of 2 per cent uranium nitrate and 2 per cent potassium ferricyanide solutions: instead of a silver-mirror production on the surfaces of spirochetes, a colorless state of those organisms, like a photographic negative, is obtained. Spirochetes in these preparations are easy to recognize because they seem to be swollen, their contours are very clear and the whole colorless body is in sharp contrast to the yellow or brown surroundings. Sometimes the borders of the spirochetes are not sharp, but show

a transitional appearance. Sometimes in focussing up and down a fine black thread is seen, while in another focal plane can be seen the negative colorless appearance.

With the use of a special preparation it was possible to show polar bodies in bacteria (typhoid cultures). A separate paper will be published concerning this phenomenon. The dark polar bodies may represent a kind of nuclear distribution.

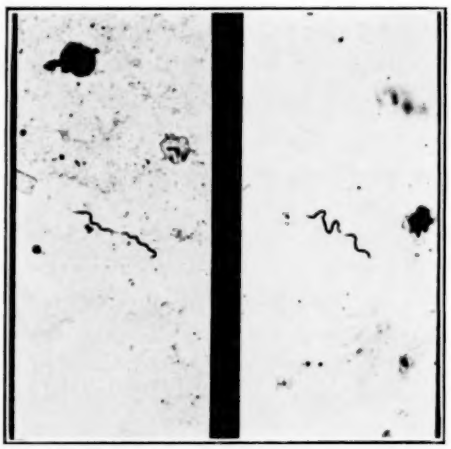

Fig. 1*A*.—Thick blood smear obtained from the tail of a mouse injected intraperitoneally, five minutes before, with *Spirochaeta recurrentis* (Duttoni). Prepared by the new method and showing transverse division of spirochetes.

Fig. 1*B*.—Showing the same as Fig. 1*A*, but in the right part of the figure a complete division of a spirochete into two individuals is seen.

The same conclusion can be made for the broken lines of the spirochetes. The black segments may represent a more nuclear substance like the polar bodies of bacteria, the colorless intervals corresponding to the other body substance.

Finally, the results of using the new method on thick smears must be mentioned. By this thick smears method thinly scattered microbes in blood could be more easily discovered, and diagnosis of doubtful cases of acute infectious diseases could be facilitated. Until now, spirochetes in thick smears could not be demonstrated by any silver-salt reduction method. Therefore,

an investigation was made to determine whether the new silver-mirror method was useful for thick smears of blood also. For this purpose I used thick blood smears from mice with relapsing fever *(Spirocheta duttoni)*, from rabbits with experimental syphilis, from guinea pigs with ratbite fever (sodoku-spirilla). For hemoglobin removal, ammonia (1 c.c. concentrated NH_4OH to 60 c.c. distilled water) must be used, washing two or three times; this procedure must be carefully watched, because ammonia tends to dissolve smears, as mentioned above. The thick smears have to be very well fixed. By this method it was possible to disclose spirochetes in low concentration in the blood stream. Giemsa and other aniline dye methods have been satisfactorily used on thick films; it may therefore be asked whether a special silver-salt reduction method is worth while. In this regard it seems to me that aniline stained specimens of thick blood smears show less color contrast between the elements of blood and the microorganisms than the same specimens prepared by the silver-mirror method. The black glaring silver mirror on spirochetes against the background of light yellowish red blood cells and blood plasma makes it easier to detect them by this stain than by aniline stains of thick smears.

It was further possible to find relapsing-fever spirochetes shortly after intraperitoneal injection of mice. (Fig. 1.) Even five minutes after the inoculation a very small number of spirochetes could be found in thick smears by the silver-mirror staining method, while dark-field and Giemsa's methods at the same time showed no spirochetes. The preparations show clearly that in living animals *Sp. recurrentis* propagates by transverse division (Fig. 1).

The silver-mirror method cannot be used on tissue sections.

SUMMARY

By copying a technical method of silver-mirror production there was obtained a silver-staining method for smears and thick films for spirochetes and bacteria—the so-called *silver mirror method.*

The method has the following advantages: it is easy to perform, stains spirochetes better than Giemsa or other aniline stains, permits use of thick films of blood or other materials, and reveals certain previously unsuspected morphologic features.

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THE ADVANTAGE OF STAINED ANTIGENS IN THE DIAGNOSTIC AGGLUTINATION TEST FOR BRUCELLA INFECTIONS*

ROGER D. MINSTER, M.SC., BOISE, IDAHO

THIS investigation was started because of the desirability of finding an inhibitory agent to be used with bacterially contaminated blood samples. For this reason considerable investigatory research has been followed, the purpose of which, was to find a material which would inhibit bacterial growth, have a low cost and at the same time not interfere with the agglutination proper.

The pseudoreactions most frequently encountered are those in which bacterial contamination plays an important part. These false reactions are most often seen in samples which are bacterially infested or in which there is a considerable degree of hemolysis. Some of these specimens give the usual appearance of contamination, namely, cloudiness, scum, and marked odor. At frequent intervals, especially during the hot summer months or during freezing weather, specimens present themselves in which there are varying degrees of hemolysis and are later contaminated due to necessary handling prior to the actual agglutination.

Five-tenths per cent phenol has been, and is used as an antibacterial agent. The use of various antiseptic solutions appearing on the market under trade names has also been suggested. No doubt all of these products have their individual merits. The type of antibacterial agent used will probably have to be adjusted to suit the needs of the individual laboratory. However, in this laboratory where a large number of samples are tested daily (varying from 450 to 900) a material possessing a high degree of effectiveness, having a low cost and at the same time having the quality of not entering into the reaction, is necessary.

Many times in routine work pseudoreactions or false flocculations accompany the use of an antiseptic solution. This is especially noticeable when phenol is used because of its coagulative effect on serums or any foreign protein in the form of hemolyzed blood cells. The more noticeable instances of these pseudoreactions are found in samples containing a considerable amount of hemolysis or a possible number of normal red blood cells which have not been removed by previous centrifugation. If no inhibitive agent is used, the amount of serum present in the agglutination tubes may be sufficient to support bacterial growth to such an extent that the reactions will be masked to the point of error.

Examination of available literature on the subject does not reveal evidence of the use of dyes in conjunction with bacterial antigens, except the dyes in the antigens used by Huddleson[1] and Welch and Marsh.[2] The dyes used by these workers were in concentrated antigens prepared for the rapid

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or plate macroscopic method of agglutination for brucellosis and not with the antigens used for the test tube method.

The purpose of this report is to suggest a method used in this laboratory for the past six months with a considerable degree of success and satisfaction.

METHODS

In this laboratory, following the use of 0.5 per cent phenol and prior to the use of combimned dyes, gentian violet (1:40,000) was employed as an inhibitive agent and was found to be quite satisfactory. However, it did not completely satisfy our need as it inhibited only the growth of the gram-positive organisms. Since the blood specimens received here are largely contaminated with *E. coli* together with common soil forms, the need for a dye having an inhibitive action on the gram-negative group was obvious. To overcome this difficulty brilliant green (1:13,000) was added to the antigen together with the gentian violet (1:40,000).

The concentrations of these two dyes is noticeably higher than necessary to inhibit the growth of the usual contaminating organisms, that is 100 times the concentration required for gentian violet, using *S. aureus* as the test organism and 10 times in the case of brilliant green with *E. coli* as the test organism (Topley and Wilson[3]). This higher concentration of the two dyes was selected for the reason that in making readings in this laboratory artificial light is used entirely and the darker color aids greatly in making the results more striking.

In the work here reported 1 per cent solutions of both gentian violet and brilliant green are used as the stock dyes. These are mixed in the ratio of one part gentian violet to three of brilliant green. Four one hundredths (0.04) cubic centimeters of the mixed dye is then added to each 100 c.c. of the prepared brucella antigen as diluted ready for use. Dilutions of serums are made in the usual manner (1:50; 1:100, and 1:200) and the antigen containing the dye added.

During the preliminary work, known reactive and nonreactive serums with varying degrees of titer were used. These known samples were then inoculated with a staphylococcus and *E. coli;* separately and in combination. These two microorganisms were used because they are most frequently encountered in our work. In the tubes in which no inhibitive agent was used, growth was sufficient, especially in the dilutions of 1:50, and 1:100, to cloud the antigen to such an extent that clear cut readings were not possible. In this instance, where reactions of 1:50 were encountered no reaction would be determinable when no antibacterial agent was used. In the protocols to which the combination of the two dyes was added to the antigen, this bacterial growth and ultimate clouding was not present and the reactions were well defined and clear-cut in all instances.

The microorganisms in the antigen containing the dye take up a sufficient amount of it to give the precipitated bacteria in the positive specimens a very marked coloration. This concentration of the mixed dyes in the final antigen is not sufficient to mask the reaction yet aids materially in the readings, and pseudoreactions are seldom encountered.

SUMMARY

The experimental evidence presented in this paper is given to aid, if possible, persons who have encountered similar conditions due to contamination of blood samples and agglutination tubes, causing pseudoreactions.

These observations were made on and are given as applicable to brucella antigens. Modifications might be used for other microorganisms.

Contaminated specimens give clear-cut results when the mixture of the two dyes, gentian violet (1:40,000) and brilliant green (1:13,000) is added to the antigen and not in cases in which no inhibitive agent is used.

The method of preparing the antigen for the diagnosis of brucella infections has been used by the author for the past six months with very satisfactory results.

The results encountered in this series of experiments would indicate that the use of gentian violet (1:40,000) and brilliant green (1:13,000) is advantageous since bacterial contamination is not encountered nor do hemolyzed specimens give the usual pseudoreactions so frequently noted when no bactericidal agent is used.

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THE DETERMINATION OF HEAT IN THE GUINEA PIG*

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FROM the time of the earliest of the senior author's experiments[1] until the present, he and those associated with him have depended upon behavioral responses in determining the time of heat or sexual receptivity in the guinea pig. Reliance has been placed upon these responses partly because they can be elicited so easily,[2] and partly because they indicate so much more accurately than the vaginal smear picture the period when the female will accept the male.[3]

In consequence of our dependence upon this sign of heat, data have been obtained and experiments have been possible that would have been impossible of realization had it been necessary to rely upon the vaginal smear technique. We refer to the change in growth rate of the graafian follicle at the beginning of heat,[4] determination of the rôle of the corpus luteum hormone, progesterone, in producing heat,[5] the effect of ovum age upon the course of gestation and development,[6] studies of the ovarian condition and the character of heat,[7] the

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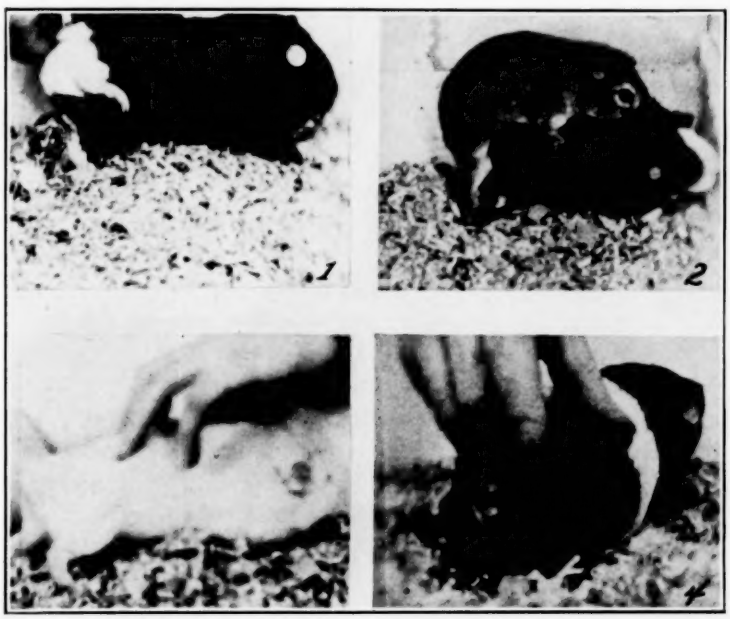
effect of a variety of hormones upon follicular growth rate,[8] and other work not yet published.

Unfortunately, if we may judge from the experience of Scott[9] who attempted to employ this method of detecting heat for an embryologic investigation, difficulties may arise which we have not experienced or which we have been easily able to overcome. The explanation may be that whereas we have described the behavioral responses prior to and during heat,[2] we have never described in detail the method which has enabled us to depend upon them so completely. It seems desirable therefore to do so now.

Our general colony of females has always been maintained in wire mesh cages with wooden frames about 40 by 30 inches in area, 16 inches high and



Figs. 1 to 4.—*1*, Repelling, kicking reaction given by female guinea pig fingered when not in heat. *2*, Mounting activity characteristic of proestrum and early part of heat. *3*, Typical heat response showing arching and straightening of back and elevation of pudenda. *4*, Dilatation of vulvar region shown during heat.

open at the top. Ten to twenty animals are kept in a cage. For convenience in handling the animals, the cages are raised 24 inches off the floor on wooden supports.

During an experiment every animal is examined three or four times daily for the open vaginal orifice, either about eight in the morning, five or six in the afternoon, and ten or eleven in the evening, or at six-hour intervals regularly throughout the twenty-four hours of the day. As soon as the membrane is found ruptured, the animals are isolated for observation in cages of the same type. They are then fingered at regular intervals until the heat response is given (Figs. 1 to 4) or until the membrane has closed. When observations are continuous, the animals are fingered every half hour, day and night. When observations are periodic, the animals are fingered only three or four times a day, but always the first thing in the morning and the last thing at night.

In this way the investigator who is not free to examine the animals at more frequent intervals can confine his efforts to those periods when the incidence of heat is high.[2]

While reviewing the data referred to in the 1935 article, it was found that when those summarized in Fig. 1 of that article were discussed, the impression was given that the percentages of animals in heat at different hours of the day refer to the percentages of animals from an entire colony. Actually what is shown is the frequency of heat at different hours of the day compiled from the total number of recorded heat periods. Consequently, the frequency of heat at each hour in an unselected population of healthy, sexually mature animals would be somewhat less than that given because some animals occasionally miss a heat period and a few do not come into heat at all. For practical purposes, however, the difference is not great and the data indicate sufficiently accurately what may be expected.

If the membrane closes and heat has not been detected, either of two things has happened. The animal has not been in heat or the period of heat has been missed by the investigator. In either case the animal is returned to the cages containing animals with closed membranes. There she may be left for twelve days without daily examination or she may be examined several times a day until the vaginal orifice is again open. In behavioral studies the latter procedure is desirable; in much embryological work the former is sufficient.

When the colony is composed of a large number of animals being observed over several consecutive cycles, time and effort can be saved if the animals whose vaginal orifices have closed during each two- or three-day period are kept together until twelve days have elapsed and examinations are resumed. This procedure establishes a classification of animals with respect to the time of the next expected heat which is useful in planning experiments scheduled for the near future.

It has been our experience that when a reasonable familiarity with the heat response has been acquired, three or four careful observations daily over a period of twenty days are sufficient for the detection of heat in approximately 75 per cent of a colony of animals. If observations are continued beyond this time, most of the animals missed during the first cycle should be found in heat during the next cycle.

Three minor deviations from normal behavior may cause some difficulty. As Young, Dempsey and Myers[2] and Scott[9] have noted, animals sometimes come into heat before rupture of the vaginal membrane has occurred. If the mounting activity characteristic of the proestrum and the early part of heat is displayed and observed (Fig. 2), the detection of heat is easy despite the presence of a closed membrane. On the other hand, if no mounting occurs or is observed, the heat period probably will be missed. Fortunately, the occurrence of heat prior to rupture of the membrane is relatively infrequent.

The second cause for confusion is the occasional elicitation of the copulatory response before the animal will permit copulation.[2] In our experience this false response, unlike the normal response, cannot be obtained repeatedly; furthermore it is not given for long and heat follows soon.

The third abnormality is the "split-estrus," described as an initial heat period of one or more hours' duration followed several hours later by a second heat period.[2] For reasons which are not known, the split-estrus is more common in animals in which heat tends to be long, and often it is repeated from cycle to cycle (unpublished data). Its occurrence, we now believe in something less than 4 per cent of the cases, does not have any serious effect on the reliability of the method.

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A CONTINUOUS INTRAVENOUS INJECTION APPARATUS FOR UNRESTRAINED ANIMALS*

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UNTIL recently the intravenous administration of experimental fluids to animals in this laboratory has been made by the needle puncture of a superficial leg vein, the fluid being supplied from either the modified Marriott cylinder or through the Woodyatt pump. Such methods are very suitable for shorter periods of injection or during injections in which the animal may be restrained or anesthetized. However, prolonged continuous experiments and the hope of more normal conditions have necessitated the use of unanesthetized animals which were allowed the complete freedom of the cages.

Our original intention was to use the constant intravenous apparatus described by Jacobs (1931). Although this apparatus gives a remarkably constant rate of flow, it is complicated. Consequently, the Jacobs apparatus has been so greatly modified and simplified that the apparatus described here resembles the Jacobs' only in that the catheter insertion into the jugular vein is somewhat similar.

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Apparatus.—Essentially the apparatus consists of a swivel unit, constant pressure cylinder and a drop trap. A speedometer cable and thick-walled rubber tubing lead to the collar of the animal. A No. 14 soft-rubber urethral catheter is used as a jugular cannula (or vein tube). The entire apparatus is suspended from an overhead beam, the cage being placed directly below.

The swivel, which is the important unit in protracted experiments, allows the animal perfect freedom (see Fig. 1). It serves both as a supporting and rotary mechanism and is constructed from a cone and ball-race from the rear axle of a bicycle. The supporting member consists of a ¾ inch iron rod threaded at the lower end and fastened securely to an overhead beam at the

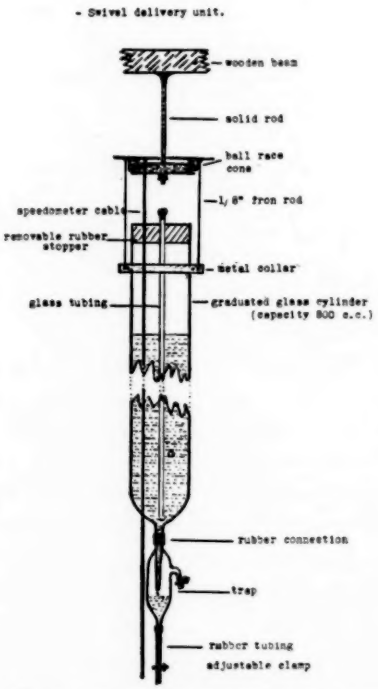

Fig. 1.—Diagram illustrating the rotary delivery system. The delivery tube and cable, shown at bottom of diagram, pass through the cage roof to the animal.

upper. A lock-nut holds the cone firmly on the lower end of the iron rod. The ball-race is placed directly above the cone and rotates on it. A large metal washer is soldered to the upper surface of the ball-race casing from which the constant pressure cylinder and speedometer cable are suspended (see Fig. 1).

The fluid reservoir, a modification of the Marriott bottle (Hall, Ettinger and Banting, 1936) is held in position by means of ⅛-inch iron rods attached above to the large metal washer and below to a galvanized iron collar. The collar is firmly clamped on the cylinder (diagram). Strips of rubber inner tubing are placed between the clamp collar and the cylinder. The cylinder is graduated in cubic centimeters and has a capacity of 800 c.c.

Thick-walled pressure tubing fastened to the speedometer cable with adhesive tape, except near the animal's neck, conveys the injection fluid to the

animal. A "G" clamp placed just below the drop trap allows for alterations in injection rate (observed in trap). The speedometer cable, long enough to allow the animal to lie down (for complete freedom) is soldered below to a metal plate on the animal's collar and above to the large metal washer. It is important to have the conducting system of greater length than the cable, in order that tension is not exerted on the catheter in the neck. Any rotation of the cable causes the ball-race to rotate on the cone. The cylinder, being firmly fastened to the ball-race, rotates with it. By such an arrangement rotation of the animal causes the entire delivery system to rotate, thus alleviating any possible twisting or kinking of the rubber delivery tube.

Procedure.—Using aseptic technique, one external jugular vein is exposed under local anesthesia (novocaine). The vein is ligated above and clamped below and a longitudinal slit made between the two. The clamp is then taken off and the catheter introduced through the slit toward the heart for about five inches. Five cubic centimeters of sterile saline are injected through the catheter and a temporary glass plug inserted in the open end. This procedure minimizes the chance of clotting within the catheter previous to beginning the injection. Three purse-string sutures are placed around the catheter and vein, each including some subcutaneous tissue between the suture and vein wall. Strips of connective tissue with one end intact, are bound and sewn to the slit through which the catheter enters. The neighboring subcutaneous connective tissue is scarified to promote growth. The skin is sutured in the usual manner. Using the above operative procedure, we have been able to administer solutions continuously for as long as three weeks without leakage.

The free end of the catheter is connected to the delivery pressure tubing by means of a small glass adapter, this junction being strengthened with adhesive tape. About five inches of the catheter lies inside the vein in use. The remainder, including a small portion of the pressure tubing, is curved convexly toward the head and taped securely to the animal's neck. This prevents kinking and allows the delivery tube attachment to the dorsal aspect of the animal's neck. The neck is then well bandaged. The collar, with the speedometer cable attached, is placed in position over the bandage. Sufficient laxity is provided between the cable and delivery tube close to the animal's neck to prevent any pull on the catheter should the collar slip. (This procedure prevents tension on the catheter in the vein.) To guard against entanglement with the animal when standing, strips of elastic are fastened to the delivery tube and cable. If the animal lies down, stretching of the elastic allows for the necessary adjustment. If pressure tubing with an outside diameter of 5/8 inch is used, a simple spring wire ironing cord shortener may be used in place of the elastic strips.

CONCLUSION

During the injection, the animal is allowed complete freedom of its cage. Food and water can be given daily or more often if necessary. The dogs appear interested and respond readily to external stimuli (tail wagging, etc.). A sloping sub-floor enables the urine (voluntary elimination) to be collected

in a container placed beneath the cage. This normal behavior indicates that no discomfort is suffered. Under such conditions experimental animals differ from the normal only in the physiologic reaction produced by the injected fluid.

The advantages of this method are:

1. The apparatus is inexpensive and easily constructed.
2. Little attention is necessary.
3. The rate of injections can easily be observed (in drop-trap) and altered if necessary.
4. The injections may be continued for weeks.
5. Sterile solutions can be used throughout with little fear of contamination.
6. The animal is neither restrained nor anesthetized.

Postmortem examinations of dogs having received intravenous injections by this method continuously for over three weeks have revealed a thick tube-like sheath of connective tissue containing the catheter.

Acknowledgment.—Sincere appreciation and thanks are expressed to Mr. L. C. Brock for his indispensable assistance in this work.

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AN APPARATUS FOR PROLONGED STIMULATION EXPERIMENTS IN UNANESTHETIZED ANIMALS*

G. W. Manning, M.A., and G. E. Hall, M.S.A., M.D., Ph.D., Toronto, Canada

DURING a recent investigation in this laboratory (Manning, Hall and Banting, 1937), it was necessary to stimulate the vagus nerve in dogs for long periods of time. The methods described in the literature were quite suitable for short periods during which the animal was restrained or anesthetized. However, in experiments where stimulation was carried out for as long as eight days (twenty-four hours per day), it was necessary to use a method which would allow the animal complete freedom of its cage. With this in mind and the desire for more normal conditions, the apparatus to be described in this paper was constructed.

Essentially the apparatus consists of a sequence timer, inductorium and a slip-ring-contact, from which the stimulating wires and a speedometer cable lead through the cage roof to the animal (Fig. 1). The important part of this apparatus is the slip-ring-contact by means of which the animal is able to turn about at will without twisting the electrode or lead-in wires.

The slip-ring-contact consists of an inner and outer unit. The inner unit (Fig. 2) is a ¼ inch iron shaft threaded at both ends for about 1 inch.

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Mounted on the iron shaft and held in place by a set screw is a bakelite rod, on each end of which is a ¼ inch brass band. A ⅛ inch hole is drilled from the lower end of the shaft up to the level of the upper brass band; through this hole two insulated wires, soldered to the upper and lower brass bands, respectively, leave the shaft. The brushes, made of brass gauze, are part of the outer unit (Fig. 3). This inner unit is held in position within a casing (Fig. 3), and is rotated within this unit on a ball race at either end. The cones are locked firmly in position on the shaft by means of lock nuts.

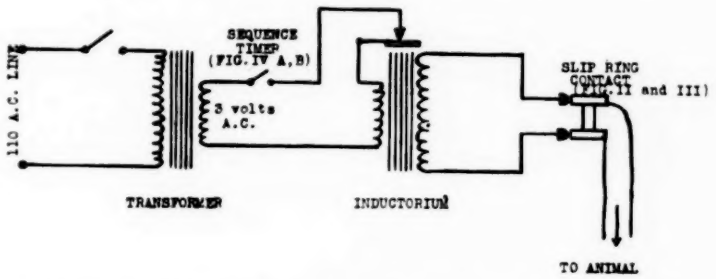

Fig. 1.—Diagrammatic representation of complete stimulating circuit.

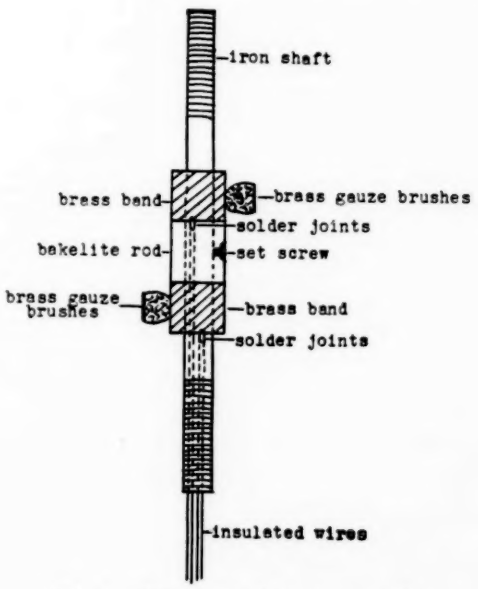

Fig. 2.—Slip-ring-contact, inner unit.

Between the upper and lower flanges on the outer casing is mounted a bar of bakelite. Fastened to the bakelite bar are two binding posts from the base of which, on opposite sides of the casing, are fastened two ¼ inch strips of spring brass. The brushes (Fig. 2) (soldered to the inner side of the brush holders) are held in contact with the inner unit through two ½ inch square holes drilled directly over the inner brass bands on opposite sides of the casing (Fig. 3). A speedometer cable, soldered to the lower lock-nut, completes the slip-ring-contact which is fastened to the roof of the animal's cage.

The speedometer cable, approximately 30 inches in length, is soldered, at its lower end, to a metal plate on the animal's collar. The insulated wires run parallel to the speedometer cable and are fastened to it at intervals by adhesive tape. A shortening effect, to safeguard against any possibility of the animal becoming entangled in the wires, is obtained by a simple ironing cord shortener. When the animal is standing, the wires and cable are pulled upward; when the animal is lying down, they are automatically adjusted by the stretching of the spring. When the animal turns around, the torsion effect on the speedometer cable rotates the inner unit. Through contact of the brushes with the inner brass bands a continuous flow of current is permitted.

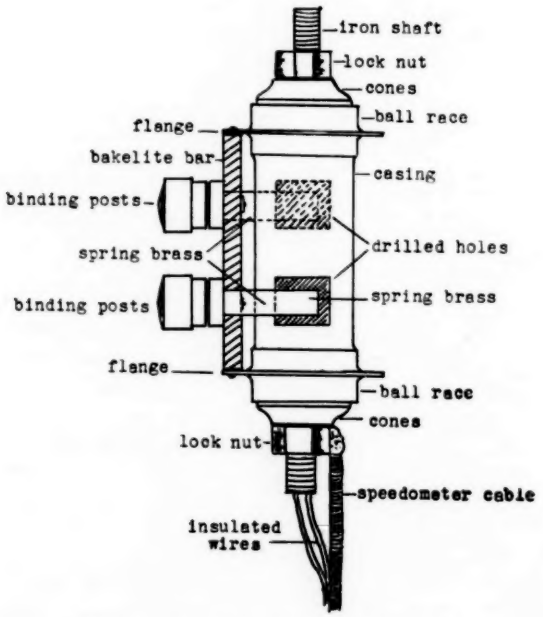

Fig. 3.—Slip-ring-contact, outer unit.

For stimulating purposes the current was taken from the 110 volt, 25 cycle A.C. line to a small transformer connected so as to deliver 3 volts. This was led through an inductorium to activate the primary. In series with the primary coil of the inductorium was a specially designed sequence timer, the details of which are shown in Figs. 4 and 5. As the large wheel rotates, the metal lugs striking the wire prongs of the rotary switch unit make and break the current which activates the primary coil of the inductorium. By adjusting the distance between the metal lugs the ratio of "stimulation on" to "stimulation off" can be varied. Thus nerve fatigue due to continuous stimulation is eliminated.

The electrodes used were similar in type to the shielded electrode described by Ettinger, Hall and Banting (1936), except that the rubber foot was ¾ inch long, and insulated soft-stranded flexible wires (Fig. 6) were used in place of single lead-in wires.

Under aseptic conditions the vagus nerve was exposed and the recurrent laryngeal nerve cut. The rubber foot shown in Fig. 6 was fitted around the nerve and closed with three interrupted black silk sutures. The wound was closed and a light gauze bandage placed around the animal's neck to serve

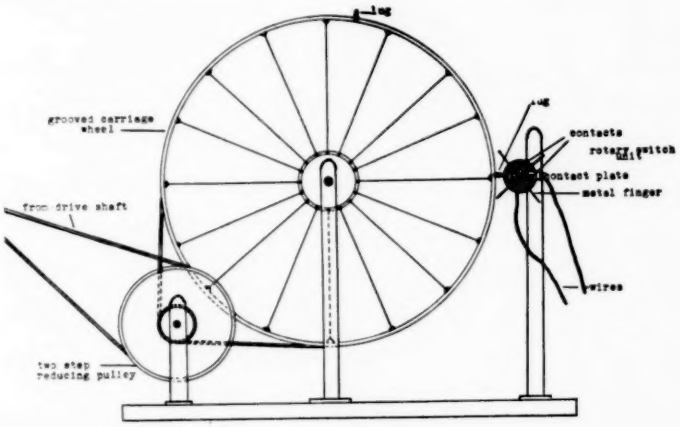

Fig. 4.—Sequence-timer, side elevation.

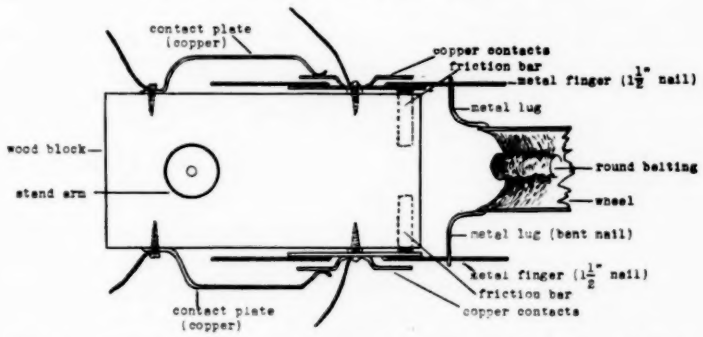

Fig. 5.—Rotary switch unit of sequence-timer, viewed from above.

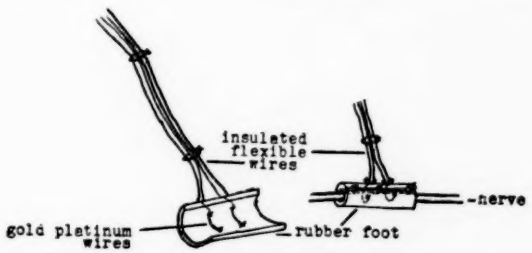

Fig. 6.—Shielded electrode.

both as a dressing and to hold the electrode lead-in wires in place. This bandage was held in place by adhesive tape. The collar, with its cable attachment, was then fitted over the gauze bandage and stimulating wires from secondary coil connected to the electrode lead-in wires.

By means of this apparatus we have been able to stimulate the vagus nerve in dogs for as long as eight days. The animal, allowed the freedom of its cage, eats, drinks, and sleeps normally.

The apparatus is inexpensive and easily constructed, since the important unit, the slip-ring-contact, can be made from an ordinary bicycle hub assembly.

Acknowledgment.—Appreciation is expressed to Mr. L. C. Brock for his technical assistance in the construction of this apparatus.

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AN IMPROVED TECHNIQUE FOR BILIARY DRAINAGE*

FILIP C. FORSBECK, M.D., LANSING, MICH.

IN CONNECTION with the diagnosis and release of typhoid carriers it has been necessary to obtain numerous bile specimens. Since September, 1935, a technique has been developed which has markedly decreased the time necessary to obtain bile.

In 1889 Boas[1] called attention to the possibility of using a stomach tube to collect bile. While of historic interest, the method is unsatisfactory because the bile is usually mixed with gastric juice. The fact that the presence of gastric juice makes a bile specimen unreliable for the diagnosis and release of typhoid carriers has been recently emphasized by Forsbeck and Hollon.[5]

In 1908 Einhorn[2] described a metal bucket tied to a thread which was carried into the duodenum by peristalsis. This was a great contribution, enabling bile to be obtained nonsurgically. Einhorn himself, however, pointed to the difficulty of obtaining serial samples and of obtaining considerable amounts of bile uncontaminated with gastric juice. The following year he[3] further improved duodenal drainage technique by passing along the thread of the duodenal bucket a tube through which bile could be withdrawn. This he called "the aspiration catheter." Finally, in 1910, Einhorn[4] described his "duodenal pump" which in principle is generally used at the present time.

The same year Gross[6] suggested a modification of Einhorn's technique. He also pointed out the frequency with which the gastric juice is mixed with bile. Jutte[7] in 1912 suggested that drinking water after the duodenal tip has reached the pyloric end of the stomach was an advantage. Palefski[8] described a tip twice as heavy as the Einhorn tip with which he was able to procure bile in from one to two hours. In his experience it took two to four hours to get bile with the Gross[6] tube and five to twelve hours with the Einhorn[4] tube. In 1914 Rehfuss[9] described a tip with which he obtained bile in from one to two hours. This tip, now generally used, is the only one with which I have had experience. There appear to be no properly controlled observations indicating that one tip is better than another.

*From The Michigan Department of Health.
Received for publication, May 21, 1937.

Irrespective of the tip used, the technique has been essentially the same. The tip is passed to the cardiac end of the stomach and then it is left to time and chance for the tip to be carried into the second portion of the duodenum by peristalsis. In the past I have used a technique essentially as described by Rehfuss and Nelson.[10] The details will not be discussed except so far as it is necessary for the description of my modification.

It should be pointed out that usually considerable time elapses from the procurement of a gastric juice-bile mixture to the procurement of bile.*

MODIFIED TECHNIQUE

This technique makes use of gravity in conjunction with peristalsis by the simple expedient of extending the stomach with water. This permits the duodenal tip to be rapidly moved to the pyloric end of the stomach.

While sitting down, the patient swallows the duodenal tube until the tip reaches the stomach as indicated by the ease with which air or fluid may be forced through and withdrawn from the tube. The stomach is then distended by having the patient drink as much water as he can reasonably tolerate, after which the tube is passed to the lower end of the stomach in a few seconds. Using a 20 or 30 c.c. syringe, the water is withdrawn, together with what gastric juice and particles of food may be present. The water not only permits the rapid passage of the tip through the stomach, but also apparently causes a backflow of bile into the stomach. A specimen consisting mostly of bile and positive for *B. typhosus* has been obtained occasionally in a few moments after the tip has reached the cardiac end of the stomach. Such a specimen, however, may not be satisfactory for the purpose intended.

The patient then lies on his right side while an additional 5 to 15 cm. of tube are passed at the rate of about 1 cm. per minute until bile is obtained. I am tentatively of the opinion that if bile is not obtained in one hour, time will be saved by withdrawing the tip to the cardiac end of the stomach and repeating the procedure from that point.

In a series of 54 bile drainages, the mean interval for the procurement of a satisfactory bile specimen was 38.8 minutes. The time necessary to pass the tip to the cardiac end of the stomach averaged five minutes. This compares with the one to two hours which seems to be the general experience. Satisfactory biles were obtained in from five minutes to two hours and forty-nine minutes in the above series. In nine instances bile was procured in less than fifteen minutes, while in only five cases was more than one hour necessary. In two cases the tip was withdrawn to the cardiac end of the stomach after failure to procure bile in sixty and one hundred and two minutes, respectively. In one case bile was then obtained in eleven minutes, in the other in sixty-seven minutes.

In three instances there was complete failure to get bile. The percentage of failures was certainly not greater than with the previously used method.

*Judging from the routine bile specimens received by the Michigan Department of Health for bacteriologic diagnosis, no great effort has been made to obtain specimens essentially bile. It has been found[5] that at least for the diagnosis of *B. typhosus* (and presumably the same would hold true for other organisms sought in the bile), it is necessary to secure a bile specimen meeting certain standards. The specimen should be clear, viscous, amber, and neutral or alkaline. While pathologic biles may vary from this, ordinarily it is wise to suspect that a gastric juice-bile mixture rather than a pathologic bile is being examined.

Even when cases are included in which no bile was obtained, the median case required but twenty-five minutes for the procurement of bile after the tip reached the stomach.

In 28 instances in which record was kept, backflow of bile into the stomach occurred in 25, or 89 per cent. The average time for bile stained gastric juice to be obtained after the tip reached the stomach was less than eight minutes. The specimens varied from those with a tinge of bile to those approaching a satisfactory bile specimen.

Specimens obtained while the tip is in the lower end of the stomach do not necessarily consist of gastric juice or of a gastric juice-bile mixture. In thirteen instances an acceptable bile specimen was obtained while the tip was obviously in the stomach.

SUMMARY

A modification of the usual duodenal drainage technique has reduced the time necessary for the procurement of bile to an average of thirty-nine minutes.

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CHEMICAL EXTRACTION WITH A SIMPLE AUTOMATIC SHAKER*

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ADEQUATE agitation of mixtures in the analytical laboratory is always a problem for several reasons: (1) Many reactions demand thorough mixing or the maintenance of a finely divided suspension for extraction purposes; (2) mechanical shakers are too costly for the majority of laboratories; and (3) manual shaking is too wearisome to insure uniformity or reproducibility of experimental conditions.

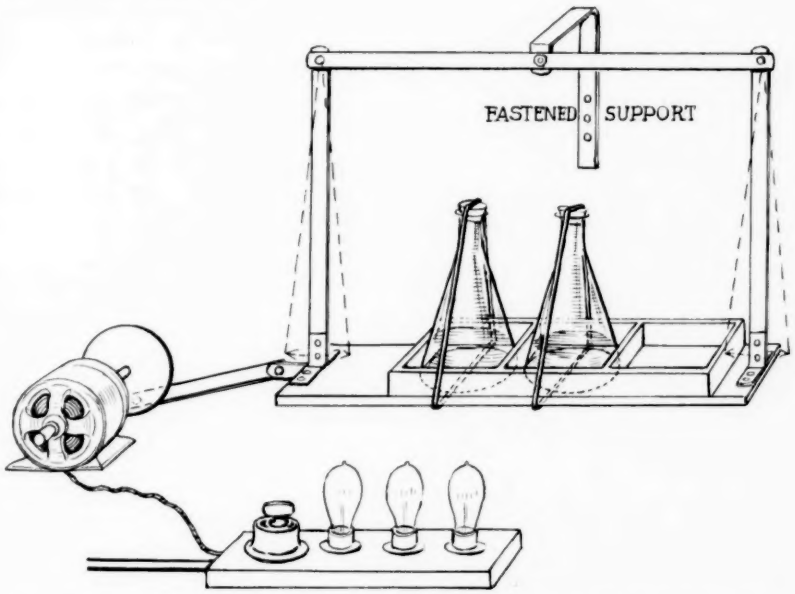

Fig. 1.—The Holtz automatic shaker with the light bulb system substituted for the rheostat.

The satisfaction which the Holtz automatic shaker has given in the Endocrine Laboratory of the Department of Gynecology of the New York Post-Graduate Hospital warrants its description for the benefit of other laboratories compelled to assemble equipment through ingenuity rather than by purchase of unduly expensive apparatus.

The shaker depicted in the illustration has been employed for the past two years for the extraction of estrin from blood by the senior author's[1] modification of the Frank-Goldberger procedure. This involves agitation of a 500 c.c. Erlenmeyer flask containing 40 c.c. of blood (previously desiccated

*From the Department of Gynecology, New York Post-Graduate Medical School and Hospital, Columbia University.

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with 30 to 40 gm. of anhydrous sodium sulfate) and 100 c.c. portions of ether for twenty-minute periods. The apparatus is so simple that the diagram almost describes it. Rubber bands (regulation size 64) are employed to secure the flasks to the wooden tray which has three compartments 3⅞ inches square (internal measurement) with walls ⅜ inch thick and 13/16 inch deep. The support is clamped either to a wall or window-sill for use on a conveniently located table. The shaker should be about 1¼ inches from the table surface. The metal uprights measure 13⅜ inches and the top bar is 18¾ inches with ⅛ inch thickness. The arm to the motor is 8 inches long and of somewhat thicker metal. The circular cam attached to the motor shaft is 3⅜ inches in diameter, the point of attachment to the drive shaft being 1⅜ inches from the center of the cam. The rotation of the cam moves the drive shaft backward and forward thus giving a to-and-fro motion of the tray, which produces agitation of the flasks. The most suitable material used in the metal supports for the tray consists of ¾ inch by ⅛ inch scrap iron. The whole unit is put together with nuts and bolts.

Mechanical power is furnished by a 1/30 H.P. motor operating on 110 volts with a current of one ampere (G. E. motor employed was obtained second-hand for less than four dollars). At first this was used with a rheostat, but a battery of lamps in series has proved more practical. The speed is regulated by the number of bulbs, one or more being used according to the load on the shaker. For many purposes a single 60-watt carbon-filament lamp is ample. When necessary, a 40-watt Mazda bulb is also switched in.

We wish to express our grateful appreciation to Miss Marjorie Mattice for her kind assistance.

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27 East 93rd Street
242 Trumbull Street

A SIMPLE METHOD FOR DEMONSTRATION OF SPIROCHETES IN FROZEN SECTIONS*

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THE process of using gum mastic as a protective colloidal substance in the silver impregnation of spirochetes was first used in 1922.[1] Five years later Dieterle[2] used it also in developing a separate method. During the last seven years a shorter modification of the method described in 1922 has been used as follows:

1. Fix tissue thoroughly in 10 per cent formalin. Fresh specimens of small blocks should be fixed for at least twelve hours or longer. When a faster procedure is wanted, keep the block eight hours in hot formalin (55° to 60°).
2. Wash in running tap water according to the size of the block, twenty minutes to one-half hour.
3. Cut frozen tissue at 25 to 30 μ.
4. Wash twice in distilled water.
5. Transfer sections into absolute alcohol and
6. Into the following mixture:
   25 c.c. of 6 per cent solution of gum mastic in absolute alcohol.
   10 c.c. of 4 per cent solution of uranium nitrate in absolute alcohol.
   15 c.c. of absolute alcohol. Filter through double filter. For six to eight minutes.
7. Wash sections in 3 or more changes of distilled water and stir them until milky clouds or gummy strings have completely disappeared.
8. Transfer sections in 0.1 to 0.15 per cent distilled water solution of silver nitrate and heat slowly in the open dish over the asbestos net until bubbles appear. Wait until sections cool. The whole procedure takes about thirty-five minutes.
9. Wash twice in distilled water.
10. 95 per cent alcohol one to two minutes.
11. Transfer sections in 3 per cent absolute alcoholic solution of gum mastic for two minutes.
12. Repeat the seventh step of this method.
13. A 5 per cent aqueous solution of hydroquinone is made. To 100 c.c. of this solution add 6 drops of the 6 per cent alcoholic gum mastic solution. A milky appearance is produced. This fluid has to be filtered through a double filter. Then transfer sections into the filtered solution. Heat to just before boiling and let sections cool. The whole procedure lasts about ten minutes.
14. Wash in distilled water.
15. Dehydrate by transferring the sections to 95 per cent alcohol, absolute alcohol, clear and mount in the usual way.

The gum mastic solution has to be very clear without any suspended particles. Three bottles are used to prepare the gum mastic solution. In the first bottle the solution is kept three days, shaking the bottle every day several times. Then the solution can be filtered through a double filter into the second bottle and two days later in the third bottle, through a double filter also.

*From the Louisiana State University Medical School, Department of Pathology and Bacteriology, and Wayne University College of Medicine.

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The whole staining method lasts about one hour. Only 4 different substances are necessary, gum mastic, uranium nitrate, silver nitrate, and hydroquinone. Because of the low concentration of the silver nitrate the amount of silver nitrate substance is less than in other silver impregnation methods.

By using thicker sections the mixture of gum mastic and uranium nitrate in Step 6 of this method has to be changed. Thicker sections (50 to 60 μ) do not need as much gum mastic. For the use of thicker sections the mixture may be composed as follows:

10 c.c. of 6 per cent solution of gum mastic in absolute alcohol.
10 c.c. of 4 per cent solution of uranium nitrate in absolute alcohol.
30 c.c. of absolute alcohol.
In Step 11 the gum mastic solution has to be of 1 per cent.

By using this method the results have been very satisfactory. The chief advantage of this method is the reduction of time, the simplicity of carrying out the procedure and its reliability. It can be used in combination with other stains, for example, with Scharlach-R, safranin, fuchsin acid, and eosin. Another advantage of this method is the impregnation of spirochetal débris in phagocytic cells and free in the tissues. By this method spirochetes have been found in primary and secondary stages of syphilis and in cases of general paresis. In acquired syphilis in the adult spirochetes were found in syphilitic lesions of the heart* and the larynx.* In 2 congenital cases spirochetes were found in the intestinal tracts* and other organs.

By this method spirochetes could be demonstrated in experimental syphilis of rabbits, in mice and rats inoculated with *Spirochaeta duttoni* and guinea pigs inoculated with *Spirilla morsus muris* (ratbite fever).

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*I am indebted to Doctors von Haam, Ogden, Pearson and Tuta for placing at my disposal the above mentioned material of congenital and acquired syphilis.

AN IMPROVED RING MAKER AND AN EMULSION PIPETTE FOR THE KLINE TEST*

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SINCE Kline[1] published his description of a flocculation test for syphilis, there have been two papers describing improvements in the apparatus used for the test. Varney[2] described an apparatus for rotating serum antigen mixtures, and Magath[3] described an emulsion pipette, slide holder, paraffin bath, and a serum antigen rotating machine. In this laboratory, an improved ring maker and a simplified emulsion pipette somewhat similar to that of Magath have been found to lessen greatly the labor of preparing for the test.

The ring maker consists of six pairs of brass curtain rings 16 mm. in diameter which may be purchased at any 5 and 10 cent store. Two rows of three rings each are placed on a glass plate and soldered together. Another six rings are superimposed upon the first six and are soldered to them for strength. A needle holder clamp is then soldered to the top center of the unit and the needle holder is screwed into place. If, when a trial is made, there is a slight unevenness in the ring print, the ring maker is gently rubbed down on a large flat file. No wrapping or coating of the rings is necessary. For use, the paraffin is heated to 130° C.; the ring maker is warmed in it; the excess paraffin is rapidly drained; and the rings are pressed on a slide cleaned in the usual manner. When enough paraffin has drained onto the slide and before it has had time to harden, the ring maker is lifted vertically off, leaving behind six paraffin rings each 15 mm. in diameter.

The emulsion pipette consists of a stainless steel syringe needle which is prepared by cutting off the bevel while the stylet is in place and honing down the rough edges. If too many drops of emulsion are delivered by a needle, the number can be decreased by spreading the tip with the point of a pin. If too few drops are delivered, it will be necessary to use a smaller needle. We found a No. 26 gauge needle to be suitable for the exclusion antigen emulsion and a 24 gauge needle for the diagnostic emulsion. With a tuberculin syringe and a needle prepared as above, the required amount of antigen emulsion can be rapidly and accurately dropped into the serum.

SUMMARY

An improved ring maker consisting of soldered brass curtain rings and an emulsion pipette utilizing a syringe and needle are described.

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*From the George Williams Hooper Foundation for Medical Research, Medical Center.
Received for publication, May 24, 1937.

A MULTIPLE PIPETTING MACHINE*

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WITH the widespread application of the tube agglutination test in the diagnosis of Bang's disease in cattle and pullorum disease in poultry, the setting up of the serologic tubes with the proper amount of antigen suspension has become an economic problem in many laboratories. In order to reduce the time consumed in this operation, a mechanical pipetting machine was devised which is capable of filling about 140 tubes per minute. This machine may be constructed at a reasonable cost.

MATERIAL

Wooden Frame.—This was made of white pine boards having the following dimensions: two pieces, 2 by 6 by 15 inches; one, 2 by 6 by 12 inches; one, 2 by 3 by 12 inches; five, 1 by 4 by 24 inches; and three, ½ by 2 by 12 inches.

Hardware.—An assembly of 4 rocker arms with tappet adjustments and 4 push-rods from a discarded valve-in-head automobile motor, and 8 tire valves, were obtained from a service garage. One jackshaft assembly (Driver), 2 bronze bearings with supports (Driver), 4 V-type pulleys (one 10 inches, one 6½ inches and two 1¾ inches), 2 V-type belts (one 24 inches and one 34 inches), 12 carriage bolts (5/16 inches by 2½ inches), 8 angle irons, 2 straight irons, 1 box of wood screws (No. 8, length 1½ inches), 1 electric motor (1/10 H.P., 1120 r.p.m.), and 1 electric switch with extension cord, were purchased from an ordinary hardware store.

Four special coiled steel springs, 8 push-rod bearings and eccentric shaft with counterbalance were obtained from a machine shop. The eccentric shaft was made from a steel shaft, 1¼ by 12 inches, by grinding the two ends down to a diameter of ½ inch for a distance of 2 inches. The center of the ½ inch portions was located ¼ inch from the edge of the original shaft so as to obtain a lift of ¾ inch when the shaft was rotated.

Glassware.—This was as follows: 10 feet of Pyrex tubing ¼ inch O.D.; 1 wide-mouth bottle, capacity 2.5 liters; and 4 Luer syringes, capacity 5 c.c., with openings of from 1.5 to 2 mm., to permit rapid flow of antigen.

Miscellaneous.—Twelve feet of good grade medium wall rubber tubing, 3/16 inch inside diameter, were used for connections, and several small strips of lead were employed in constructing the valves.

CONSTRUCTION

The frame was constructed as shown in Figs. 1 and 2. The dimensions of the base were 1 by 12 by 24 inches, and of the upright 2 by 12 by 15 inches. The upright was firmly attached to the base with four rugged angle irons.

*From the Department of Animal Diseases, Storrs Agricultural Experiment Station. Received for publication, May 31, 1937.

The eccentric shaft, push rods with bearings, and rocker arm assembly were then installed and aligned by a machinist.

The jackshaft and motor were mounted, as shown in Fig. 1.

The syringes, valves, U-tubes, delivery tube assembly, antigen reservoir and connections were constructed as follows:

Syringes.—The tops of the plungers were covered with rubber to avoid breakage. Special steel springs, to supply lifting power, were placed around the plungers before the syringes were placed in block W1. A film of light oil, such as 3-in-1, was placed on the plungers of the syringes. Suitable notches were cut in block W1 to permit easy removal of the syringes.

Adjustment of the volume of antigen delivered by each syringe was made possible by mounting a rigid steel rod (not shown in diagram) on block W1

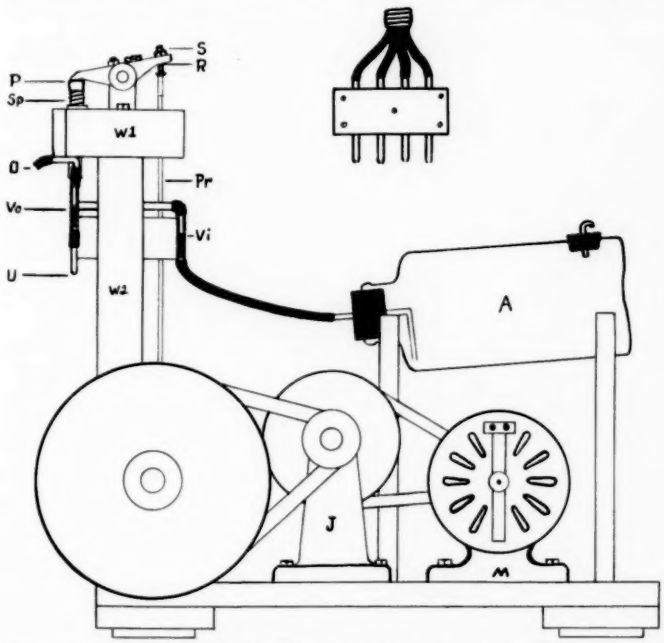

Fig. 1.—Side view of multiple pipetting machine. *A*, antigen reservoir. *J*, jackshaft. *M*, motor. *P*, plunger of syringe. *Pr*, pushrod. *O*, outlet. *R*, rockerarm assembly. *S*, adjustable screw. *Sp*, spring coiled around plunger. *U*, U-tube with sidearm. *Vi*, inlet valve. *Vo*, outlet valve. W_1, wooden block 12 by 6 by 2 inches. W_2, wooden block 15 by 6 by 2 inches. *Inset*, delivery tube assembly.

under the rocker arms just in front of the push rods. After removing the air from each unit by working the plungers by hand, the screws *(S)* were adjusted so that each syringe delivered 2 c.c. of antigen.

Valves.—Brass valve stems removed from discarded inner tubes were prepared for use by cutting off the base and grinding down the outside to a diameter of about ¼ inch. The springs on 8 new Schrader valve cores (long) were removed and small strips of lead wound around the shaft of the valve cores. Both the inlet and outlet valves were mounted in an upright position (Fig. 2), so that the weight of the lead facilitated the closing of the valves.

U-Tubes With Side-Arms.—Four U-tubes with side arms were constructed in a manner which permitted connections with the syringes and inlet and outlet valves. Holes were drilled in the upright *(W2)* for the side arms. The side

arms were connected to the inlet valves, as shown in Figs. 1 and 2. One end of each U-tube was then connected to a syringe, and the other to an outlet valve (Fig. 2).

Delivery Tube Assembly.—Four glass tips were mounted between two pieces of plywood, as shown in the inset in Fig. 1. The distance between the tips was the same as the distance between the centers of a series of four agglutination tubes arranged in wooden blocks. Four 30-inch lengths of rubber tubing were connected with the outlet tubes and the tips. The tubing was then wound with heavy cord.

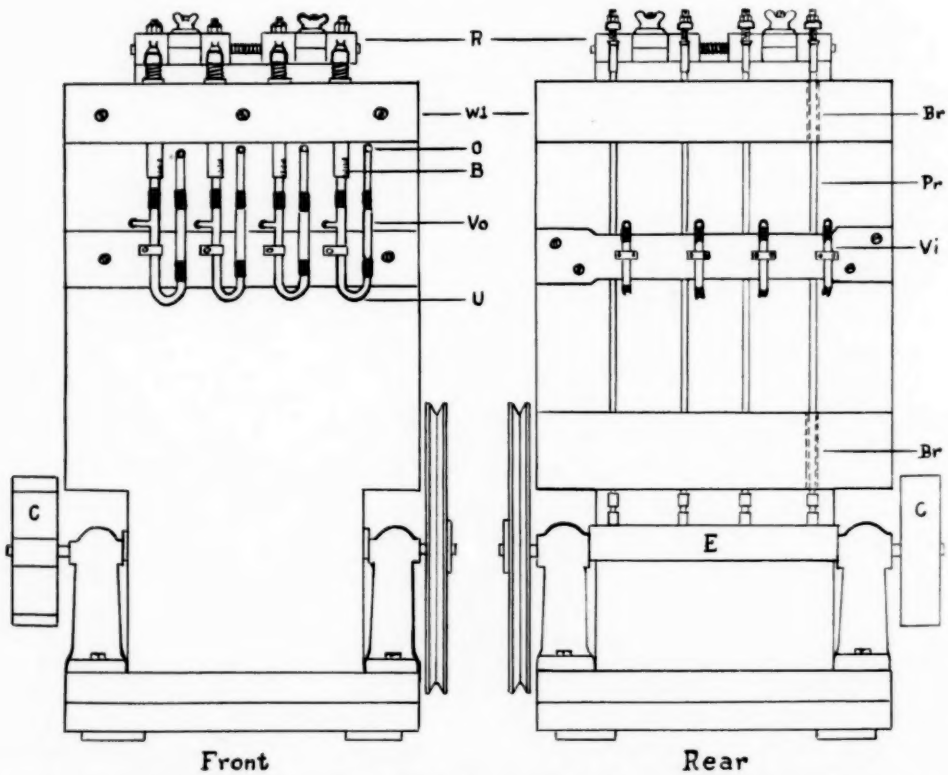

Fig. 2.—Front and rear view of multiple pipetting machine, with antigen reservoir, jackshaft and motor removed. *B*, barrel of syringe. *Br*, brass bearings. *C*, counterbalance. *E*, eccentric shaft. *O*, outlet connected with delivery tube assembly. *Pr*, pushrod. *R*, rockerarm assembly. *U*, U-tube with sidearm. *Vi*, inlet valve. *Vo*, outlet valve.

Antigen Supply.—A large wide-mouth bottle *(A)* was mounted on two uprights at a level slightly below the inlet valves (Fig. 1). Four glass tubes were placed in the rubber stopper in the mouth of the antigen bottle. The tubes were then connected to the four-inlet valves with rubber tubing. The antigen was filtered previously through coarse filter paper, in order to remove small particles of agar and bacterial clumps.

Miscellaneous.—The appearance of the machine was greatly improved by inclosing the entire unit in a case constructed of plywood. Sections of the plywood were supplied with hinges, to permit easy access to the working parts. The completed unit was mounted on four large rubber stoppers.

ANALYSIS OF RENAL CALCULI*

JONAS KAMLET, B.Sc., BROOKLYN, N. Y.

IN THE chemical examination of renal calculi, the following procedure has been found to be of great value due to its accuracy and simplicity.

A complete determination of the chemical constituents of a calculus can be made on as little as 10 mg. of material. Small calculi, usually termed sand or gravel, may be examined in toto. Larger calculi should be transected and each of the concentric layers subjected to a separate analysis. If possible, 100 mg. of powdered material should be obtained by scraping from each layer with a blunted scalpel.

1. Description of the calculus as to shape, size, color, weight, and appearance on transection. Thus: "Specimen submitted is a smooth, oval, pale yellow renal calculus about the size of a pea, weighing 4.826 gm. On section, an inner concentric layer of brownish yellow crystals are observed," or "Specimen submitted comprises twelve small, rough, amorphous gray renal calculi varying in diameter from 1.5 mm. to 3.0 mm., weighing totally 844 mg."

2. The specimen for analysis is divided into two equal portions. One portion is reserved for the detection of cystine, xanthine, urostealith, fibrin, cholesterol and indigo. The other portion is macerated to a fine powder in a clean, dry porcelain mortar and added to 50 c.c. of 10 per cent hydrochloric acid (sp. gr. 1.05) in a small Pyrex flask. Effervescence at this point indicates the presence of carbonate ion. The mixture is gently boiled for five minutes, filtered through ashless filter paper and the filtrate is evenly distributed into five clean 6 by 1 inch test tubes.

3. One tube is rendered faintly alkaline with 10 per cent NaOH solution and 5 c.c. of Nessler's reagent is added. A brown turbidity or sediment (the iodide of Millon's base) indicates the presence of ammonium ion.

4. One tube is rendered faintly alkaline with 10 per cent NaOH solution and then cautiously re-acidified with dilute acetic acid. One cubic centimeter of arsenophosphotungstic acid and 4 c.c. of 5 per cent NaCN solution is added. A blue color developing within sixty seconds indicates uric acid or urate ion.

5. One tube is rendered faintly alkaline with 10 per cent NaOH solution and then reacidified with dilute acetic acid. A white turbidity or precipitate indicates the presence of calcium oxalate. If no turbidity results after standing for ten minutes, 5 c.c. of a saturated ammonium oxalate solution is added. A white precipitate or turbidity at this point indicates the original presence of Ca ion only (probably as $CaCO_3$).

6. One tube is rendered ammoniacal with strong aqua ammonia and 1 c.c. of a 0.5 per cent solution of p-nitrobenzeneazoresorcinol in 95 per cent alcohol is added. A blue color developing within thirty seconds indicates the presence of magnesium ion.

*From the Department of Laboratories, Israel-Zion Hospital.
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7. To the last tube is added 2 c.c. of acid molybdate reagent (equal parts of 10N H_2SO_4 and 7.5 per cent Na_2MoO_4 solution) and 1 c.c. of dilute $SnCl_2$ solution (1 c.c. of a solution of 10 gm. of $SnCl_2$ in 25 c.c. conc. HCl diluted with 200 c.c. of water). A blue color developing within sixty seconds indicates the presence of phosphate ion.

8. The residual half of the specimen for analysis is agitated with 10 c.c. of chloroform. An indigo calculus will turn the chloroform blue. The chloroform is then decanted from the insoluble sediment.

9. A few drops of the chloroform extract are evaporated on a glass slide and stained with Sudan III. Residual fat globules on the slide indicate a urostealith calculus.

10. To the remainder of the chloroform solution is added 5 c.c. of acetic anhydride and 0.5 c.c. of conc. sulphuric acid. A Liebermann-Burchard reaction (play of colors ending in a naphthol green shade) indicates the presence of cholesterol.

11. The insoluble sediment from Step 8 is divided into three parts. One part is dissolved in nitric acid and cautiously dried over the naked flame. A yellow residue which turns orange on the addition of 10 per cent NaOH solution and deep red on warming, indicates the presence of xanthine.

12. To the second part of the insoluble sediment from Step 8, add 2 c.c. of Millon's reagent and heat. A positive protein reaction (red precipitate) indicates the presence of fibrin in the calculus.

13. To the last part of the insoluble sediment from Step 8 add 2 c.c. of conc. aqua ammonia and agitate for two or three minutes. One drop of the resultant solution is allowed to evaporate on a slide. If cystine is present, six-sided platelets will separate out on the spontaneous evaporation of the ammonia. For a confirmatory test, dissolve 5 to 10 mg. of powdered calculus in 5 c.c. of N/10 HCl, add 2 c.c. of 5 per cent NaCN solution, mix and let stand ten minutes. Add 1 c.c. of a 0.5 per cent aqueous solution of 1,2-naphthoquinone-4-sodium sulphonate and 5 c.c. of a 10 per cent solution of Na_2SO_3 in N/2 NaOH solution. Mix and let stand for thirty minutes. In the presence of cystine, a reddish brown color forms which will turn deep red on the addition of 1 c.c. of a 2 per cent solution of $Na_2S_2O_4$ in N/2 NaOH solution.

During the past three years, 644 renal calculi were examined by this method at our laboratories. Of these, 472 were of the uric acid-ammonium urate variety, 78 were of the magnesium ammonium phosphate (triple phosphate) variety, usually containing smaller amounts of calcium phosphate and calcium oxalate, 88 were of the calcium oxalate variety (of these 12 contained smaller amounts of calcium carbonate). Four were pure cystine calculi. One fibrin calculus, one xanthine calculus but no indigo or urostealith calculi were encountered.

DEPARTMENT OF REVIEWS AND ABSTRACTS

ROBERT A. KILDUFFE, M.D., ABSTRACT EDITOR

LEAD POISONING, Comparison of Punctate Basophilia and Ratio of Large and Small Lymphocytes in the Diagnosis and Prevention of, Shields, D. O. Med. J. Australia **1**: 535, 1937.

The stippled cell count, the ratio of large to small lymphoid cells, and the concentration of lead in the urine are considered in relation to the diagnosis and prevention of lead poisoning.

The comparison between the former two methods has been based on three different lines of investigation: (1) comparison of the distribution of the values in three clinical classes; (2) correlation coefficients between the values of each factor and those of the corresponding urinary lead concentrations in Class A; (3) the occurrence or otherwise of unusual variations in the values.

The results indicate that the ratio is more closely correlated with the clinical condition than are the total stippled cell count or the coarsely stippled cell count, and it is more useful as a guide both to the prevention and diagnosis of lead poisoning.

The inadvisability of attempting to adjudge the severity of cases by laboratory standards alone is emphasized.

The results of 295 urinary lead examinations are summarized.

When the subject is examined while still at work in the lead hazard, or within a few days of leaving it, one may expect to find lead poisoning with disability when there are present the following: (a) total stippled count, 2,500; (b) coarsely stippled cell count, 1,000; (c) ratio of large to small lymphoid cells, less than 1.5; (d) concentration of lead in urine in milligrams per liter, 0.15 and over; (e) coarsely stippled cell count divided by the ratio of large to small lymphocytes, 800.

TISSUE: Biopsy Studies of Human Endometrium, Rock, J., and Bartlett, M. K. J. A. M. A. **108**: 2022, 1937.

The suction biopsy is a very useful method of endometrial diagnosis, better, the authors believe, than the punch biopsy or curettage. The specimen should be taken from high on the anterior or posterior wall of the corpus, for only here are cytologic changes complete and progressive.

Two phases of endometrial development during the menstrual cycle are cytologically distinguishable: the proliferative, attributable to estrogen, which shows well marked differences between its earlier stage and its subsequent development; the second, the secretory phase, attributable to both estrogen and progestin. During this phase, mutations in the glands and their epithelium in the stroma and its cells are characteristic of successive days and make possible a day by day appraisal of corpus luteum activity.

Such dating of the endometrium on an arbitrary scale as for a twenty-seven-day cycle with ovulation on the fourteenth day is useful for determination of ovulation, for diagnosis in menstrual disturbances and for evaluation of endocrine therapeusis.

TRICHOMONADS, Incidence of in Vagina, Mouth and Rectum, Bland, P. B., and Rakoff, A. E. J. A. M. A. **108**: 2013, 1937.

Our study of the incidence of Trichomonas from the vagina, mouth and rectum of 200 women employed wet smears, stained slides and culture methods.

Vaginal infestation with Trichomonas was common, occurring in 23.5 per cent of the group. Buccal infestation occurred in 16.5 per cent of the patients, while intestinal trichomoniasis was comparatively rare, being present in only 1.5 per cent of the women.

It was demonstrated that the wet smear method is the most efficient for the diagnosis of *Trichomonas vaginalis*, while the culture method was much superior for the detection of the intestinal and buccal trichomonads.

Women harboring vaginal trichomonads did not show an appreciably higher percentage of buccal or intestinal trichomonads than was noted for the group at large. Only one woman (0.5 per cent) harbored all three organisms.

Rectal contamination was regarded as an improbable source of vaginal infestation in view of the rarity of intestinal trichomoniasis among women with trichomonas vaginitis.

Of three patients harboring intestinal trichomonads, only one was positive for *Trichomonas vaginalis* despite the fact that the flagellates could be regularly demonstrated in the feces over long periods of time.

Auto-infestation with buccal trichomonads was also considered an unlikely source of vaginal infestation because the dual incidence of these organisms was not more than would be expected from chance distribution.

A comparison of the incidence of trichomonads from the bowel and mouth indicates that intestinal infestation probably does not result from ingestion of the buccal forms.

Unlike the intestinal and buccal trichomonads, *Trichomonas vaginalis* was not cultivable at room temperature and was considered physiologically different.

TRICHINAE, Encysted, Incidence in Private Practice and Bearing on the Interpretation of Diagnostic Tests, Magath, T. B. J. A. M. A. **108**: 1964, 1937.

Although this is the only recorded series of microscopic examinations made on a relatively large number of unselected patients in private practice, it is clear that they, although perhaps not so frequently infected with trichinae as are patients from economically lower walks of life, nevertheless often harbor the parasite. Exposure must be common.

From available knowledge it is evident that, in the United States, from 10 to 20 per cent of the adult population have acquired trichinae. Although some of those persons have experienced more or less typical symptoms of trichinosis, most of them never have had any signs suggestive of the infection having taken place. This makes intepretation of the skin and precipitin tests difficult, since it is not known whether immunity follows mild or even severe infection with this parasite.

Eosinophilia of more than 10 per cent, and especially rapidly rising eosinophilia, should suggest trichinosis. If, in addition, the skin test is positive in a dilution of antigen of 1:10,000, if a history of eating undercooked pork is obtained, and if typical symptoms are present, the diagnosis would appear almost certain. The final proof must still remain the demonstration of the worms in the body of the victim.

In spite of the fact that trichinosis has not received widespread consideration in the United States, one must conclude, on the basis of studies already made, that it is one of the major public health problems in this country and that there is still need of emphasizing the importance of cooking pork thoroughly before it is consumed.

TUBERCULIN TEST, Technical Factors Affecting the, Nelson, W. E., Seibert, F. B., and Long, E. R. J. A. M. A. **108**: 2179, 1937.

Evidence is presented on the heat stability of tuberculin and the tendency of tuberculin to remain adherent to glassware and rubber. A certain proportion of tuberculin-positive subjects react to the injection of physiologic solution of sodium chloride from syringes previously used for tuberculin and then simply washed and sterilized. The use of pipets and rubber stoppers, previously employed for tuberculin and subsequently cleaned by ordinary methods, may introduce into supposedly tuberculin-free mediums sufficient tuberculin to produce occasional reactions.

The clinical importance of these facts is apparent. When Schick tests were made with syringes previously used for tuberculin and cleaned by ordinary methods, falsely positive

Schick tests, in reality tuberculin reactions, were often obtained. The danger in the employment of tuberculin syringes in routine allergy tests, as with pollen proteins ,is also apparent.

Phenol in concentrations of 0.25 to 0.5 per cent in physiologic solution of sodium chloride does not cause a reaction simulating the tuberculin reaction.

The most effective methods of cleaning syringes to destroy all traces of tuberculin are boiling in soap solution and prolonged immersion in sulfuric acid-postassium dichromate cleaning fluid. It is probably safer and more practical, however, to use syringes never before used for tuberculin whenever a tuberculin reaction might confuse the result, as in the Schick test or in various protein allergy tests.

ALBUMINURIA, Febrile, Welty, J. W. Am. J. M. Sc. **194**: 70, 1937.

Forty patients have been studied from the standpoint of urinary albumin level before, during, and after an uncomplicated fever.

True febrile albuminuria of varying degree occurs in over 75 per cent of cases. This increased level is carried over into the postfever stage.

The mechanism may be that of relative renal anoxemia following cutaneous vasodilatation.

POLIOMYELITIS, Results of Prophylactic Picric-Acid Alum Spray in. W. P. A. Information Service.

Partial success in the prevention of poliomyelitis through treatment with a picric acid-alum solution has been announced by Dr. Charles Armstrong of the United States Public Health Service in Transactions of the Southern Branch, American Public Health Association.

Dr. Armstrong, who is co-developer of the solution, has made a number of findings and recommendations as a result of the wholesale experiments on humans carried on last year during the poliomyelitis epidemic in Alabama, Tennessee, and Mississippi. His chief recommendation is that investigations proceed in the effort to find an ideal solution and the most effective manner of applying it.

These were the first experiments carried on on a large scale with human beings, although Dr. Armstrong, together with Dr. W. T. Harrison of the United States Public Health Service, had previously experimented with monkeys saving 24 out of 25 which, after having been sprayed with the solution, were infected with poliomyelitis virus. They lost 20 or 26 monkeys which had not received the spray before infection.

When the Alabama outbreak of infantile paralysis came last summer, it was decided to carry out field trials, but, Dr. Armstrong says in his report: "It was made clear that the evidence as to protective action of the proposed spray was based entirely upon animal experimentation and was not to be considered of proved value in the prevention of poliomyelitis in man."

When the seriousness of the outbreak in Alabama became apparent, all of the 30,000-odd W. P. A. workers and their families were sprayed every other day at first, and then once a week. In addition 91 W. P. A. nurses were detailed to counties without health units, not only to spray the W. P. A. workers and their families, but to help care for the victims of the disease.

Dr. Armstrong kept careful laboratory check of the results in Birmingham and the surrounding territory of Jefferson county.

Physicians in Alabama, as well as Tennessee and Mississippi, where there also were infantile paralysis outbreaks, particularly were requested to report any cases of untoward results of the use of the spray. They reported only seven such cases among an estimated 2,000,000 persons who were sprayed in the three states. These seven cases of untoward results were not of a serious nature.

In making his compilations, Dr. Armstrong considered every person, however inadequately sprayed, to be in the sprayed class. Also, every case that developed after spraying, how-

ever faulty or inadequate the method, was attributed to the sprayed group, although it was assumed that it took two weeks for the spray to become effective and cases developing within that time were excluded from the sprayed group.

On this basis, seven cases were found within the sprayed group, while theoretically 11.4 cases might have been expected, these data being based on weeks of life and the number of cases in the nonsprayed group. All of the seven had been home-sprayed.

After a general discussion of the problems involved in the experiments, Dr. Armstrong made the following findings and recommendations:

"1. Chemicals capable of blocking the olfactory route of infection must be thoroughly applied to the nasal vault if maximum protection is to be secured.

"2. Many children actively resist and thus render spraying difficult.

"3. Sympathetic parents, unfamiliar with the anatomy of the nose, are not, as a class, qualified to administer intranasal prophylactics properly.

"4. A house-to-house survey revealed complaints by 885 from among 4,631 sprayed individuals. Headache, temporary nausea, burning of nostrils, symptoms of head cold, irritated throat, and irritation of eyes, in the order named, were the most usual complaints. Had the applications of the chemicals been more uniformly thorough more unpleasant consequences might have developed.

"5. Seven instances of hypersensitivity or of idiosyncrasy to the drugs were reported from the whole epidemic area.

"6. The actual incidence of poliomyelitis in the group sprayed by whatever method was somewhat less than the calculated incidence based upon the rate in the unsprayed control group (16:21.7) (Birmingham area).

"7. The occurrence of cases in persons who had sprayed for several weeks in the advised manner throws question upon the method as employed.

"8. In the face of an epidemic of poliomyelitis the people can be relied upon to employ any simple, inexpensive prophylactic method or promise.

"9. It seems probable that the most effective method of application, as well as the most ideal solution, has not been found yet. Investigation work should therefore be continued."

HUMAN MILK: Its Lysozyme Content and Bacterial Count, Blatt, M. L., and Kessler, H. Am. J. Dis. Child. **53**: 768, 1937.

It is a truism that for reduction of the mortality of infants, especially premature infants, human milk is invaluable.

The present study deals with the factors affecting the bacterial count of expressed human milk and was undertaken to determine whether such milk might be safely fed raw to infants and particularly to premature infants. Both lysozyme and the indigenous bacteria are destroyed by boiling. It seems desirable to preserve these elements. This can be done only if the milk is fed in the raw state.

Wet nurses were carefully selected for quantity of milk and physical health. They were examined physically and tested serologically. Their milk was examined bacteriologically to determine what were the prevailing organisms, including hemolytic streptococci. Smears of material from the pharynx were made on hemolytic plates.

The breasts were made surgically clean, and the milk was collected by aseptic technic. The authors have shown that the bacterial count is dependent on careful preparation of the breasts and on the method of expression and that if the milk is expressed manually the bacterial count is initially low. Furthermore, they have shown that holding human milk in a refrigerator for as long as seventy-two hours does not increase the bacterial count significantly. The fact that the low initial count does not show an important increase is evidence of the high lysozyme content of breast milk. There is an advantage in implanting the normal gram-positive bacillus of breast milk in those infants who must be sustained by human milk. This can be done by using the milk in its raw state.

The authors believe that one of the important factors in the reduction of the mortality of premature infants at the Cook County Hospital has been the feeding of raw milk, collected as here described. Breast milk not conforming to the requirements laid down in making this study should be boiled before using.

Lysozyme exists as an active bacteriolytic agent in human milk and is greatly reduced by boiling. It varies in amount.

In the presence of adequate amounts of lysozyme, the bacterial count of milk kept at 9° to 12° C. for from twenty-four to seventy-two hours is lower than that initially recorded.

The higher the initial lytic index, the greater is the reduction in the bacterial count during refrigeration.

Staph. albus was the predominating organism observed.

In confirmation of the observations of Schlaeppi, manual expression of breast milk was proved preferable. Milk obtained by that method has a lower bacterial count than milk collected by means of a hand-operated or an electric breast pump.

The lysozyme content apparently is not influenced by the duration of the period of lactation between the second week and the twelfth month.

The lytic index did not vary significantly in the different portions of milk obtained from one woman at a single sitting.

Hence, it would appear, according to the facts presented in this study, that raw milk with a bacterial count of less than 10,000 per cubic centimeter which has been obtained under aseptic precautions from women free from tuberculosis, syphilis, and acute infections and having normal breasts may be pooled, kept in a refrigerator for from twenty-four to seventy-two hours and safely fed to premature or young infants.

GONOCOCCUS COMPLEMENT FIXATION, Significance of, as a Diagnostic Aid in the Study of Arthritis, Warren, C. F., Hinton, W. A., and Bauer, W. J. A. M. A. **108**: 1241, 1937.

The results in 614 gonococcus complement fixation tests on the serums obtained from 316 patients with various types of arthritis indicate that the test is an important diagnostic aid in routine study of arthritic patients because:

1. Of 125 tests done on the serums from the 74 cases of proved or probable gonorrheal arthritis, 81.6 per cent gave positive reactions at some time during the course of the arthritis.

2. Of the tests done on the serums from the 52 proved cases of gonorrheal arthritis, 80.7 per cent gave positive reactions at all times.

3. Only 18.4 per cent of all the tests made in the 74 proved and probable cases of gonorrheal arthritis were negative.

4. Of 239 cases representing other types of arthritis, 91.6 per cent gave consistently negative reactions.

5. A positive reaction obtained in a case in which the history is not consistent with a diagnosis of gonorrheal arthritis is not significant. In cases in which the history is consistent with a diagnosis of gonorrheal arthritis, a positive test will be correct in 90 per cent of the cases. In about 20 per cent of the cases the reaction will be negative.

If the technical imperfection of the method employed can be improved, it is possible that the gonococcus complement fixation test will approach the Wassermann test in its degree of accuracy.

The method employed in this study follows:

1. *Antigen.*—Four Torrey strains (Nos. 8, 15, 32, and 34) of the gonococcus are grown on neutral veal, 1 per cent starch agar, for from twenty-four to forty-eight hours. This growth is washed off with 0.5 per cent phenol in distilled water and diluted according to the Hopkin method so that 2,500 million organisms are contained in 1 c.c. This suspension is shaken in a shaking machine for about sixteen hours. It is then kept in a refrigerator.

2. *Complement.*—A 10 per cent solution of guinea pig serum prepared from the mixed blood of five or more guinea pigs.

3. *Sheep's Cells.*—A 5 per cent suspension of washed sheep's corpuscles. This is based on whole defibrinated blood and not on packed cells.

4. *Amboceptor.*—Antisheep, diluted so that 0.25 c.c. induces hemolysis of 0.5 c.c. of the washed sheep's corpuscles in the presence of 0.5 c.c. of the complement. Twice this amount, or 0.5 c.c. is used in the titration and tests.

5. *Saline.*—An 0.85 per cent solution of sodium chloride in distilled water.

6. *Sensitized Cells.*—Equal parts of the 5 per cent sheep's cells and diluted amboceptor.

7. *Patient's Serum.*—Inactivated by heating at 55° C. for one-half hour.

For each day's work the following routine is carried out: complement titration; antigen titration; and the test.

The tubes are incubated at 37° C. for one-half hour and are then read. The tube in Row 1 that contains the smallest amount of complement causing complete hemolysis is chosen as the unit of complement. The 10 per cent solution is then diluted so that 0.5 c.c. contains two units. This is called dilute complement. The tubes in Row 2 are used as controls of the hemolytic system and hemolysis should not occur in any one tube.

Incubation is carried out for forty minutes at 37° C. in a water-bath, and 1 c.c. of sensitized cells is added to each tube. The tubes are incubated again for one hour and then read; 0.05 c.c. less than the largest amount of antigen that permits complete hemolysis is used in the test. If, for example, the tubes that contain 0.1 c.c. and 0.2 c.c. of antigen show complete hemolysis and the one that has 0.3 c.c. shows partial hemolysis, then 0.15 c.c. of antigen would be used in the test. This will be designated in Table III as 0.x c.c.

Saline solution is added to each tube to make the volume 1 c.c.

After the tubes are incubated for forty minutes at 37° C. in a water-bath, 1 c.c. of sensitized cells is added to each tube and then incubated for another hour.

For a satisfactory test, Tube 2 (the antigen control) and Tube 3 (the serum control) should show complete hemolysis.